AN INVESTIGATION INTO FACTORS INFLUENCING THE PRODUCTION AND DEGRADATION OF MICROCYSTINS

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A thesis submitted in partial fulfilment of the
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Declaration

This thesis, which is submitted for consideration for the degree of Doctor of Philosophy, is a record of research carried out in the School of Life Sciences, The Robert Gordon University, under the supervision of Dr. Linda Lawton, Dr Anne McPherson and Dr Hazel Wilkins. It is believed to be original except where due reference has been made and has not been presented for any other higher degree.

Douglas J. L. Graham

Abstract

Microcystins (MCYST) potently inhibit protein phosphatases and these cyclic heptapeptides are powerful hepatotoxins. They are known to be secondary metabolites produced by several species of cyanobacteria. The release of these peptides into water supplies poses a considerable threat to both humans and animal both as acute or prolonged exposure. The function and regulation of these peptides has yet to be elucidated as most results are inconclusive.

This thesis examines MCYST levels in cultures of *Microcystis aeruginosa* grown under various growth conditions, by determining changes in intracellular MCYST in relation to biomass, cell number, chlorophyll a, protein and extracellular levels. Increasing inorganic carbon through the addition of sodium bicarbonate, strongly affected MCYST levels as concentration increased MCYSTs decreased. Both the intra and extracellular data confirmed that a decrease in production had occurred, not cellular excretion. Additional investigations also confirmed the reductions were not linked to elevated sodium ions. However, growth of *M. aeruginosa* in media sparged with 5% CO_2 enriched air did not lead to reduced microcystin levels,

therefore implying that MCYST levels are only reduced by bicarbonate levels.

The persistence of these secondary metabolites in the environment is a major concern as potable water guidelines become more stringent. Identifying and understanding natural degradation processes which could be developed or used to predict degradation rates is essential, studies identified that rates of degradation for different MCYST variants are affected by the microbial diversity of the water body. However, prior exposure to multiple toxins increases a microbial population's ability to degrade complex compounds like MCYST and nodularin.

This thesis also highlights the need for developing an advance treatment approach, TiO_2 photocatalysis has relatively recently been explored for industrial application of water management although the cost of using UV is prohibitive. Therefore work developing new and novel catalysts which have equal or better activity for degrading compounds while using only visible light as the activation energy and not UV is essential. This study identified a few novel visible light catalysts the best of which (KSH Burg) rival the degradation potential of the UV photocatalyst Degaussa P25 under UV light.

Dedication

The belief of my Family

and Mr. J. Mitchell

Acknowledgments

I would like to express my sincere thanks to my supervisor Dr Linda Lawton, for her friendship, guidance, patience and for the infectious enthusiasm towards cyanobacterial toxin production throughout this project. It has been a privilege to learn from Linda both as an undergraduate and as a post-graduate student. I also extend my thanks to Dr Christine Edwards and all those in the school of life sciences who have helped in one way or another throughout my stay and made life in the department a great joy. I would like to thank my other supervisors Dr Anne McPherson and Dr Hazel Wilkins for their support. I would especially like to thank my fellow students for sharing the experience of PhD candidature especially Jackie, Russell, Eddie, Scott, Steve, Sam, Sasha, Marcus, Alistair, Stephen and Dave for their guidance and support throughout.

Last but not least I want to extend my thanks to my family and close friends for their love and support throughout my time as a student. I am indebted to Mum and Dad, not only for their love but for the opportunities they have given me along the way. Most of all I want to thank my loving partner and best friend Perdeep. She has endured this thesis as much as I have and has kept me going through the tough times. Thank you for everything.

Chapter 1

Introduction to microcystin production and degradation

1.1 Introduction

1.1.1 Cyanobacteria

Cyanobacteria are photoautotrophic prokaryotic organisms devoid of organelles such as nuclei, mitochondria, chloroplasts, golgi apparatus and endoplasmic reticulum present in eukaryotic cells (Van Den Hoek *et al.*, 1995). They are considered as one of the earliest life forms as fossilized remains of unicellular and filamentous forms attributed to cyanobacteria have been identified in sedimentary rocks formed some 3.5 billion years ago around the early Precambrian period. Additionally endolithic (growing within rock) forms that reproduced with baeocytes (a small spherical reproductive cell) are present in rocks formed 1.5 billion years ago. Heterocystous forms and other forms with true branching have appeared more recently, after the Precambrian period. These fossils of cyanobacteria are morphologically similar to their present day counterpart and many aspects regarding the rate of evolutionary changes in the cyanobacterial lineage remain unanswered (Wilmotte, 1994). These organisms and presumably their early geological ancestor's success can be attributed to various features within this group, as many cyanobacteria are able to tolerate fluctuations in temperatures of several degrees higher than

eukaryotic algae and other variation of their physiological conditions (Whitton, 1992). They are able to synthesis chlorophyll *a* and at least one phycobilin pigment (Mann, 1992).

Cyanobacteria have had many names throughout history, the most common being blue-green algae due to the similar morphology they have to eukaryotic algae and their ability to photosynthesis. Only after studies identified them as being more closely related to the prokaryotic than the eukaryotic algae, were they termed blue-green bacteria (Cyanobacteria) (Van Den Hoek *et al.*, 1995). Although they have a simple structure the, metabolic activities are compartmentalized into multienzyme granules that optimize processes such as metabolism and growth (Oberholster *et al.*, 2004). These multienzyme granules are specific for certain metabolic pathways and are located in the intracellular membrane and cell membrane within the periplasm of Gram-negative bacteria.

In cyanobacteria the process of photosynthesis typically utilizes, water as an electron donor leading to the release of oxygen into the environment. This ability of cyanobacteria to produce oxygen from water and research of fossil data, palaeontology, geology and geochemistry from the Precambrian period (Whitton, 1992), provides very convincing evidence that

suggests cyanobacteria played a major role in the oxygenation of the atmosphere. Cyanobacteria are also thought to be the evolutionary origin of plant chloroplasts, the bodies responsible for plant photosynthesis. Theorists have suggested that photosynthesizers were taken up by microbes, eventually losing their ability to function independently and became chloroplasts (Carmichael, 1994).

Cyanobacteria have been found to inhabit a wide variety of habitats with a high degree of success, they posses many physiological characteristics that are able to tolerate some of the most extreme conditions. They have been found in freshwater and in the sea, on damp soil and even in such extreme and inhospitable places as glaciers, desert and hot springs (Van Den Hoek *et al.*, 1995). They are often the first phototrophs to colonize bare areas of rock and soil, having adapted ultraviolet absorbing pigments to increase their fitness in a relatively exposed land environment (Mur *et al.*, 1999). The majorities however live in freshwater environments such as reservoirs, lakes and slow flowing streams, with the ability to grow at significant depths (with low light) as well as on the surface (with high light).

The phytoplankton of many lakes, reservoirs and rivers under prevailing environmental conditions become dominated by

cyanobacteria, resulting in higher than average biomass. This process has been classed as bloom formation, the most profound being those produced by species containing gas-vacuoles. These cyanobacteria are able to regulate their buoyancy, through alterations in the turgor pressure of gas filled inclusions. This gives cells the advantage of migrating into higher light conditions, reduces sedimentation losses and improves access to nutrient supplies (Dow *et al.*, 2000). The species commonly found in surface blooms are colonies of *Microcystis* and aggregates of filaments as in *Anabena* and *Aphanizomenon*.

Many cyanobacterial species are also able to grow on the bottom sediments of sufficiently clear water bodies, and these benthic species are thought to form coherent mats. Then during high rates of photosynthesis the oxygen produced as bubbles within the mats, is thought to be able to accumulate enough to potentially break sections off. These sections are very buoyant and float to the surface, where they are usually washed onto the surrounding banks (Mur *et al.*, 1999). Increased available nutrients under ideal growing conditions can cause major problems within many rivers, lakes and reservoirs, as they cause a natural enhancement of biological production (eutrophication). This is usually caused by high levels of

nitrogen and phosphorus, resulting in cyanobacterial surface blooms. Increased organic matter may lead to a depletion of dissolved oxygen as decay occurs, resulting in problems of fish mortality and liberation of toxic substances (Harada *et al.*, 1999). The majority of increases in nutrient levels usually occur naturally by natural soil erosion or as a result of anthropogenic activity.

1.1.2 Cyanotoxins

The presence of many cyanobacterial blooms throughout the world, in fresh, brackish and marine waters have been frequently found to be toxic. This has lead to concerns over the environmental and health implications of such bloom formations. Most cyanotoxins have been found to be more hazardous to terrestrial mammals than to aquatic boita (Sivonen *et al.*, 1999). A large proportion of research into these toxins produced by cyanobacteria has been stimulated by cases of poisoning to agricultural livestock, wild animals, dogs and human fatalities (Codd *et al.*, 1988). The main route of poisoning is following direct ingestion of surface blooms, but in a case of human fatalities in Brazil, water used in a dialysis clinic had insufficient water treatment (Kuiper-Goodman *et al.*, 1999).

There are three main groups of toxin found to be produced by cyanobacteria; these are hepatotoxic (cyclic peptides), neurotoxic (alkaloids) and lipopolysaccharides (Cornish, 2000). The main focus of this study was in the hepatotoxic group commonly associated with cases of poisonings, produced by the MCYST and nodularin families (Sivonen *et al.*, 1999). These are cyclic peptides; comparatively large peptides with molecular weights ≈ 800 – 1,100 Da and usually water-soluble, although there are a few which are more hydrophobic. The MCYSTs have a common structure consisting of three D-amino acids (alanine, *erythro*-β-methlyaspartic acid and glutaminc acid), two L-amino acids, which are variable, plus two unusual amino acids namely N-methyldehyroalanine and a hydrophobic 20-carbon chain, unique to these toxins, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda). There have been over 70 MCYSTs characterized so far (McElhiney *et al.*, 2005;Sivonen *et al.*, 1999) with variations in the amount of methlyation and demethylation, changes in the two L-amino acids and structural variation in the Adda. The naming of MCYST (MCYST) indicates the position (X or Z) and type of amino acid, for example in (Figure 1.1) position X contains leucine (L) and position Z contains arginine (R) to give MCYST-LR (Sivonen, 1996;Sivonen *et al.*, 1999).

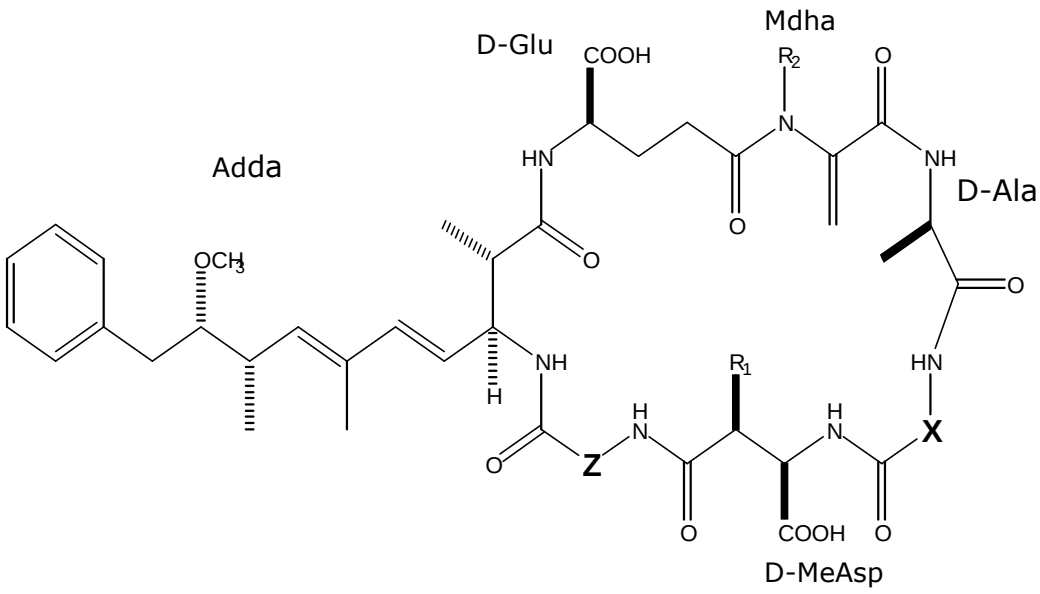

Figure 1.1
Structure of MCYST-LR with two variable amino acid positions marked with an X and Z, positions R1 and R2 depict either H (demethylmicrocystins) or CH_3 to give the different microcystin variants.

Another closely related group is the cyclic pentapeptides called nodularins, these show similar hepatotoxicity to that of MCYST and target the same organs in mammals (Sivonen *et al.*, 1999). They are produced by the genus *Nodularia*, with the exception of motuporin containing valine, characterized from the marine sponge *Theonella swinhoei* (Sivonen *et al.*, 1999). The chemical structure of nodularin (Figure 1.2) is Cyclo-(D-Masp-L-Arginine-ADDA-D-Glutamic acid-D-Mdhb), where D-Masp is D-*erythro*-β-methylaspartic acid. Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-tri-methyl-10-phenyldeca-4(E), 6 (E)-dienoic acid and Mdhb is *N*-methyldehydrobutyrine. Only a few naturally

occurring variations of nodularin have been found these are detailed in Table 1.1.

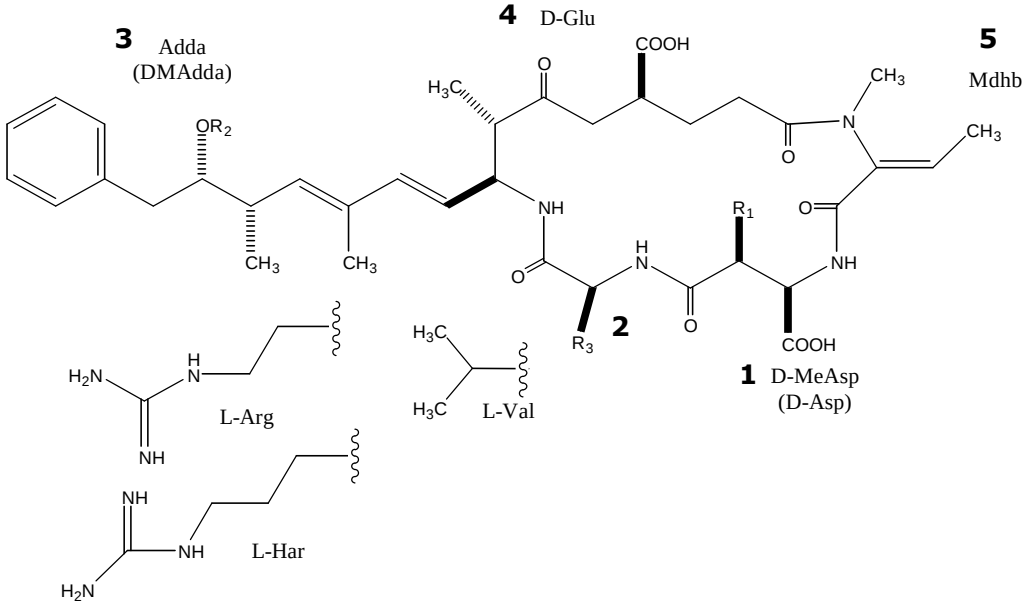

Figure 1.2
Nodularin with three amino acids that can be found in the R_3 position

Table 1.1
Through the addition of various methyl groups or amino acids to positions R_1, R_2 or R_3 of the generic nodularin structure (Figure 1.2) a variety of different nodularin variants can be formed.[Superscript are variation of to amino acids in positions 1-5]

	R_1	**R_2**	**R_3**
nodularin (nodularin-R)	CH_3	CH_3	L-Arg
[DMAdda^3] nodularin	CH_3	H	L-Arg
[D-Asp^1] nodularin	H	CH_3	L-Arg
[l-Val^2]nodularin (motuporin)	CH_3	CH_3	L-Val
[L-Har^2] nodularin	CH_3	CH_3	L-Har

The mammalian toxicity of MCYSTs and nodularins is mediated through their strong binding to key cellular enzymes called protein phosphatases (Sivonen *et al.*, 1999). They interact in a remarkably similar manner to okadaic acid (a potent tumour promoter), demonstrating high specificity with potent inhibition of protein phosphatases 1 and 2A (MacKintosh *et al.*, 1990). They are the two major phosphatases in eukaryotic cells used to dephosphorylate serine and threonine residues. The greatest threat from MCYSTs and nodularins is to human health, as an acute exposure to a high dose causes death from liver haemorrhage or failure (Falconer, 1991;Falconer, 1994). Also chronic low-dose exposure from water supplies where treatment is only able to remove the bulk of cell biomass and not free cyanotoxins, has been identified as a cause of chronic liver damage and tumour growth promotion. An example of the problems of low-dose exposure has been found in China where they have one of the highest incidences of human hepatocellular carcinoma (Yu, 1989;Yu, 1995).

1.1.3 Detection of Cyanotoxins

The detection of MCYST and nodularin can be carried out using a variety of methods that fall into two categories: bioassay and physicochemical methods. Quite often the analysis of MCYST

and nodularin requires a combination of both, although it depends on the type of sample to be analysed and the information required. The bioassays developed for cyanotoxins utilize the bioactivity of the toxins, like heptotoxicity, neurotoxicity, cytotoxicity, enzymatic activity and immunological interactions (Harada *et al.*, 1999). The mouse bioassay commonly uses male Swiss Albino mice for toxicity testing; an intraperitoneal injection of 0.1-1.0 ml^{-1} sample of cyanobacterial cell lysate (prepared in a water or physiological saline solution) is administered to the mouse. The mice are then observed for 24 hours, and then killed by an approved method and a post-mortem of the tissue carried out. The results from the post-mortem and any observed symptoms are used to determine which cyanotoxins are present. The correct cyanotoxin identification can be made very difficult in the presence of fast acting toxins as they can mask the symptoms of other toxins. The toxicity is usually expressed as LD_{50} mg cell dry weight per kg mouse body weight, where a high LD_{50} means low or no toxicity and a low LD_{50} is very toxic (Harada *et al.*, 1999).

The ability of hepatotoxins produced by cyanobacteria to inhibit protein phosphatase 1 and 2A has been exploited in order to give an accurate method for determining all hepatotoxins produced by cyanobacteria (Dow *et al.*, 2000). The inhibition of

protein phosphatase enzyme 1 and 2A is assessed by the release of radiolabelled substrate ^{32}P-phosphate (Holmes, 1991;Lambert *et al.*, 1994). The process is not only sensitive to sub-nanogram levels of toxins, but can be used to analyse many samples in a few hours. It is also possible to use it on a very wide variety of samples like fresh water, seawater and even tissue extracts, the only reservation however is, the radio isotopes require specialised equipment and regulations (Harada *et al.*, 1999). It is also possible to use a colorimetric protein phosphatase inhibition assay, in which p-nitrophenol phosphate or luciferin phosphate is hydrolyzed by protein phosphatase 2A (Harada *et al.*, 1999). Now more commonly using p-nitrophenol phosphate this colorimetric method is much safer and dose not require specialized equipment or staff trained in radioisotope handling, making it much more suitable for field analysis and routine monitoring (An *et al.*, 1994)

Cyanotoxins can also be analysed by an enzyme linked immunoabsorbent assay (ELISA) method, designed to test for MCYST and nodularin within water samples at a sensitivity of 0.05 μg / L. Polyclonal ELISA test kits are available (Envirologix. Inc) for testing MCYST; with antibodies fixed in wells of a microtitre plate. Initially calibrators, a negative control and samples are bound to the antibodies in the wells, followed by a

MCYST-enzyme conjugate that binds to the remaining antibodies. The plate is then rinsed thoroughly with ultra pure water and the bound enzyme measured colorimetrically in an ELISA plate reader, the concentration of MCYST is inversely proportional to the colour intensity (Harada *et al.*, 1999). In the past ten years a number of different ELISA methods have been developed with varying degrees of accuracy and in some cases poor cross reactivity to the different MCYST variants (Rapala *et al.*, 2002). The specificity and sensitivity of these tests are dependent on the ability of the antibodies to recognize all the MCYST variants. Recent advances in the use of recombinant antibodies expressed in *E.coli* has improved their sensitivity, selectivity and stability (McElhiney *et al.*, 2005). Recombinant antibodies are also easier to produce and can be tailored to specific variants, there are five main types of commercially test kits available; direct and indirect sandwich ELISAs, direct competitive antigen capture ELISA, or direct and indirect competitive antibody capture ELISA all available from (Biosense Laboratories AS, Thormøhlensgt. 55 Bergen, N-5008, Norway).

The physicochemical methods generally provide the ability for rapid screening of large numbers of samples, with excellent reproducibility although it generally requires significant capital investment. They also have an advantage over the bioassay methods, since they can be used for toxin identification and

quantitation at low concentrations. The concentration of MCYST or nodularin can also be analysed using UV spectrophotometery, although the main chromophore is a conjugated diene in the Adda residue, *N*-methyldehydroalanine strongly absorbs at a wavelength of 238 nm as a result of the α,β-unsaturated carboxyl group. The unknown concentration of a pure MCYST or nodularin sample prepared in methanol and blanked against methanol, can be calculated using Beer's law $A = \varepsilon l c$ where A is absorbance, ε is the molar absorptivity (in older literature it is sometimes called the extinction coefficient, this molar absorptivity varies with the wavelength of light used in the measurement), *l* is the cell path length and *c* is the concentration of solution.

High performance liquid chromatography (HPLC) is a very common analytical instrument used for the separation of polar, involatile and thermally sensitive analytes in different circumstances. The separation of compounds occurs by creation of distribution equilibria between the solid stationary phase and liquid mobile phase under high pressure. The eluting sample components can be detected by using UV detection, photodiode-array or mass spectrometry.

The methods for the separation of MCYSTs have developed over many years initially starting with isocratic elution's, however as

more MCYSTs have been identified the need for more specialised methods were required. Research by Lawton *et al.,*(1994) provided one of the most used methods to separate MCYSTs, using a reverse phase C18 silica column with a mobile phase gradient of water and acetonitrile both containing 0.05% trifluroacetic acid is employed. Mixing these mobile phases in different proportions provides a constantly changing range of polarities, which separates the MCYSTs according to their polarity, able to be detected at the MCYST characteristic maximum at 238 nm by UV detection. This provided the ability for quantitatively analyse and assess purity, although for toxins with variants containing tryptophan the maximum is at 222 nm. In situations where sample analysis requires a variable wavelength then often photodiode array detectors are best suited, as they can record spectra for each peak from 200-300 nm. Also improvements in detector hardware and spectral matching software mean they are able to detect very sight changes in chemical composition, when compared to the spectral library of standards. The HPLC analysis used in the separation of MCYSTs is also ideal for nodularin separation, with only the retention time varying.

In cases where further identification and confirmation is required the HPLC system is often coupled to a mass spectrometer; this

enables simultaneous separation and identification of the MCYSTs within a sample. The mass spectrometer can be altered in a variety of ways to optimise the ionisation of the compound and obtain the required spectral information. Fast atom bombardment (FABMS) and liquid secondary ion (LSIMS) mass spectrometry are used to obtain the compounds molecular weight (Kondo et al., 1992). The Frit-FAB LC/MS is used for separation and identification provided the analytes have different relative molecular masses (Kondo *et al.*, 1992). For small samples analysis matrix-assisted laser desorption / ionisation (MALDI) coupled to a time-of-flight spectrometer gives molecular mass and the post source decay spectra are characteristic for different MCYSTs (Erhardt *et al.*, 1997).

The availability of purified standard has been recognized as one of the major problems for sample analysis, as it makes it more difficult to identify individual components of a sample. Although there has been an increase in toxins identified over the past few years, the availability of highly pure toxins has not significantly increased in the last eight years.

1.1.4 Regulation of toxin production

Cyanotoxin production has been studied for many years in batch and continuous cultures, testing a variety of environmental factors in order to understand toxin regulation in cyanobacteria. Culture age in batch cultures and temperature are the parameters most frequently examined, followed by light, nutrients, salinity, pH and micronutrient concentrations (Sivonen, 1996). Studies mainly focused on the hepatotoxic, *Microcystis*, *Oscillatoria (Planktothrix)*, *Anabaena* and *Nodularia*; anatoxin-a producing *Anabaena*, *Aphanizomenon* and *Planktothrix*; saxitoxin producing *Aphanizomenon* and *Anabaena circinalis* (Sivonen, 1996).

The presence of nitrate and phosphate has been well documented as important factors in eutrophication of lakes and also seems to affect toxin production in cyanobacteria. Watanabe *et al.,* (1985) found that under nitrogen and phosphorus nutrient deficient conditions, the toxicity (measured as LD_{50} in mice) was found to decrease under nutrient limited conditions. Utkilen *et al.,* (1995) found nitrogen and phosphate limited conditions showed no effect on toxin production. However, other investigations into the effects of limited nitrogen and phosphate have proven to reduce the production of toxins in

M. *aeruginosa*, by about 5 fold in limited nitrogen and 4 fold in limited phosphate conditions (Sivonen *et al.*, 1999). Research into the effects of nitrogen and phosphorus limited conditions on toxin production seem to have contradicting results, although it is very difficult to compare results from other research as toxin production can be expressed against many factors such as; biovolume (Orr *et al.*, 1998), per cell (Hesse *et al.*, 2001), chlorophyll *a* (Lyck *et al.*, 1996) and biomass (Sivonen, 1990;Utkilen *et al.*, 1995). The most environmentally relevant is probably biomass as it compares the amount of toxin relative to a known weight of cells. The effects of available nitrogen on toxin regulation in cyanobacteria are however only applicable to species not capable of nitrogen fixing.

The presence of micronutrients (Al, Cd, Cr, Cu, Fe, Mn, Ni, Sn and Zn) has been tested on the regulation of hepatotoxins in cyanobacteria. LukaÈ *et al.,* (1993) studied *M*. *aeruginosa* toxins and found that the presence of metals (Al, Cd, Cr, Cu, Mn, Ni and Sn) had no effect on growth rate or toxin yield. Only the presence of Fe and Zn significantly affected the toxin yield, never inducing a change in toxin production of more than 40%. The findings by (Utkilen *et al.*, 1995) state that reducing the Fe concentration decreases toxin production, however these results

are conflicted by the findings of (LukaÈ *et al.*, 1993;Lyck *et al.*, 1996) where decreased Fe increased toxin production.

Toxin production under varying light intensities has produced many conflicting opinions, as the findings from many authors are usually not directly comparable, when growth conditions and detection methods differ. Although while studying increasing light intensities (Van der Westhuizen *et al.*, 1985;Watanabe *et al.*, 1985) both observed similar increases in toxin production in *M*. *aeruginosa*. LukaÈ *et al.,* (1995) found the ratio of toxin to protein to be independent of light intensity between 40 and 75 microeinsteins (m^{-2} s^{-1}). The effect of light intensity is an important factor in control of peptide toxin production in *M*. *aeruginosa* and changes in this physical factor can uncouple the production of toxin from general protein synthesis. Therefore, alteration of light intensity seems to be an excellent tool for further investigation of the molecular mechanism behind toxin production (Utkilen *et al.*, 1995). Research has identified that during photosynthesis plants and algae use energy in the region of the electromagnetic spectrum from 400 – 700 nm. This range has been referred to as the photosynthetically active radiation (PAR) which can be measured in energy units (watt m^2) or as Photosynthetic Photon Flux Density (PPFD) which has units of quanta (photons) per unit time per unit surface area. Light

intensity in relation to PPFD is now considered the more acceptable method of reporting light intensity among plant scientist, horticulturists, ecologists and environmental scientists. The units most commonly used are micromoles of quanta per second per square meter (μmol s^{-1} m^{-2}) as used in this thesis (McCree, 1972).

Toxin production varies between strains and species, with their highest production usually found during optimum growth conditions. Temperatures of 18-25°C usually produce the most toxins, although temperature gradients caused 2-3 fold decreases in toxin content (Sivonen *et al.*, 1999). Increasing the temperature to 32°C was found to have a significant effect on growth, but the highest toxicity was found in cells grown at 20°C, temperatures of 28°C and above caused a significant reduction in toxin by some four fold (Lehtimäki *et al.*, 1994;Van der Westhuizen *et al.*, 1985;Van der Westhuizen *et al.*, 1986;Watanabe *et al.*, 1985) all observed similar affects on toxin production through changes in temperature.

During the growth of cyanobacterial batch cultures the physiology of cells is constantly changing as a result of changing nutrient levels in the media from a state of enrichment to nutrient depletion as time progresses. These physiological

changes are thought to effect MCYST production and consequently alter the MCYST content over time. Considerable research has been carried out to investigate the effect of time in batch culture growing conditions on the levels of MCYST present. The results suggest that MCYST content is generally higher when cultures are in exponential phase and decreases as the cultures approach stationary phase and declining phases (Van der Westhuizen *et al.*, 1985;Watanabe *et al.*, 1985). This suggests that the production of MCYST is a complex process affected by changes in the cell cycle and availability or consumption of nutrients. These effects however can only be observed in batch culture as time and nutrients are not relevant in steady state continuous cultures. Although steady state cultures are an experimentally convenient way, to investigate the effect of various parameters on MCYST production and other nutrient controlled cellular physiology. Nutrient stresses however are a common factor in the life cycle of cyanobacteria as changing seasons not only bring warmer temperatures but also changes in the nutrient loading in different water bodies.

Throughout all the research into environmental factors effecting cyanobacterial toxin production, to date no research has been carried out on the effects of inorganic carbon. The concentration and speciation of dissolved inorganic carbon in the environment

is strongly linked to pH through equilibrium reactions between the species: CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} (Oliver *et al.*, 2000). The most important source being CO_2, fixed through the pentose phosphate pathway, but decreases in CO_2 increases the pH. This moves the equilibrium in favour of bicarbonate and carbonate, also able to be utilized by cyanobacteria as a source of inorganic carbon. Within the cytoplasm of the cell inorganic carbon is stored as bicarbonate at levels as high as 1000 fold that of the external concentration, facilitated by the inorganic carbon-concentrating mechanism (CCM) of cyanobacteria (Kaplan *et al.*, 1994). Investigations into the role of inorganic carbon have focused on the main cellular functions and comparisons to those found in eukaryotic cells. In this investigation one of the aims was to evaluate the effect changing levels of inorganic carbon would have on MCYST production, in relation to biomass and MCYST levels, by HPLC analysis with photodiode array detection.

The pH of many freshwater environments in which cyanobacteria dominate are often alkaline with a relatively high pH (Dwivedi *et al.*, 1994;Gerloff *et al.*, 1950). In some species like *Anabeana flos-aquae* increases in pH up to pH 10 actually cause an increase in photosynthetic rate (Shapiro, 1990). The reason cyanobacteria are able to survive in such high alkaline environments is due to their ability to maintain an almost

neutral cytosolic pH (Belkin *et al.*, 1991;Dwivedi *et al.*, 1994). Cyanobacterial dominance in high pH / low CO_3^{-2} has also been suggested by (Shapiro, 1973) to be possible as they have superior CO_2 uptake kinetics compared with other phytoplankton. While green algae tend to dominate at lower pH or when the concentrations of bicarbonate are high, thus when pH does not increase significantly in the summer cyanobacteria do not tend to dominate the phytoplankton (Shapiro, 1990). The relationship between pH and low CO_2 availability on cyanobacterial dominance in freshwater has been extensively studied by Shapiro (1990).

1.1.5 Biodegradation of MCYST

Naturally occurring MCYST has been documented by (Jones *et al.*, 1994) to persist in the environment for 9 days before degradation commenced and studies by Block et al (1992) discovered that indigenous mixed bacterial populations were capable of causing biodegradation. Bourne *et al.,* (1996) isolated a bacterium and later identified it as a new *Sphingomonas* species that possessed a novel enzymatic pathway which act on MCYST-LR one of the most common cyclic peptides produced by cyanobacteria. The process is mediated by at least three intracellular hydrolytic enzymes in MCYST degradation (Bourne

et al., 1996). These were identified by the use of protease inhibitors; initially hydrolytic cleavage of the parent cyclic peptide occurs at the Adda-Arg peptide bond by an enzyme that has been designated microcystinase. The two intermediate degradation products were also observed to be less active than the parent cyclic peptide and were much less toxic to mice at doses up to 250 µg / Kg. The ring opening of MCYST-LR by bacterial microcystinase effectively renders the compound non-toxic by reducing its binding ability with the protein phosphatase target (Bourne *et al.*, 1996).

In contrast (Jones *et al.*, 1994) suggest that a biphasic degradation occurs with the sequential induction of two separate bacterial populations. Park *et al.,* (2001) identified a bacterium Y2 with the capability to degrade MCYST-RR, -YR and LR from a heterotrophic lake. This Y2 bacterium has also been classified as a member of the genus *Sphingomonas* but was later identified as a new species or even a new genus based on its 16S rDNA sequence. The rate of degradation however is very strongly dependent on temperature with maximum rates observed at 30ºC. Other hepatotoxin degrading bacteria have also be identified, research by (Singh *et al.*, 2000) discovered a bacterial strain *Alcaligenes odorans* that was able to utilize the pentacyclic triterpenoid, lantana as a sole carbon source. Although some bacteria have been identified this area of

research is still very much in its infancy as only a few bacteria have been identified and in the natural environment the process may involve a number of different species at crucial stages for the total destruction of such peptides. Although biodegradation is a very economical process for dealing with the removal of MCYST from water bodies, in some circumstances the lack or absence of such bacteria may result in bioaccumulation. However, the environmental impact and effect of increasing MCYST degrading bacterial would have to be considered, prior to changing the diversity of the naturally occurring microbial populations. Other water treatment methods would have to be employed if such introductions could result in deterioration in the water quality and biodiversity within the water body (i.e. affecting the zooplankton or fish).

Many cyanobacterial blooms occur in lakes and reservoirs which are used for dinking water and can result in the release of cyanotoxins into the water causing major concern for the water treatment bodies. The WHO has an established guideline for the level of MCYST-LR in drinking water of 1µg / L maximum concentration. Many water treatment processes actually increase MCYST levels through cell lysis, releasing the cytosolic contents in to the surrounding water and inevitably increase the levels of MCYST. The removal of such compounds can be costly and

difficult to achieve in conditions of high water throughput. Methods such as ozonation, chlorination and photochemicals (Hrudey *et al.*, 1999) have been used and are constantly being developed, for the breakdown of unwanted compounds.

1.1.6 Photocatalytic degradation of MCYST

In the past three decades the destruction of pollutants by titanium dioxide (TiO_2) photocatalysis has been investigated, the process involves the promotion of reactions by a light activated catalyst not consumed during the reaction. The catalyst is activated by the illumination and activation of oxidizing species which initiate the destruction of compounds when in solution. This process has been well documented as a method for the destruction of hazardous waste products.

The processes of oxidation and reduction are able to occur in semiconductors due to their configuration of overlapping electron orbitals that are closely packed continuous bands. These bands are separated by an energy gap (Eg) which is unoccupied by orbitals and defined by the energy difference between the bottom edge of the conductance band and the top of the valence band. The conductance band is formed by unoccupied orbital's while the valance band is composed of

occupied orbital's and when illuminated by light (hv) of sufficient energy to bridge the band gap an electron (e^-) is promoted from the valance band to the conductance band leaving a positive hole (h^+) in the valance band figure 1.3.

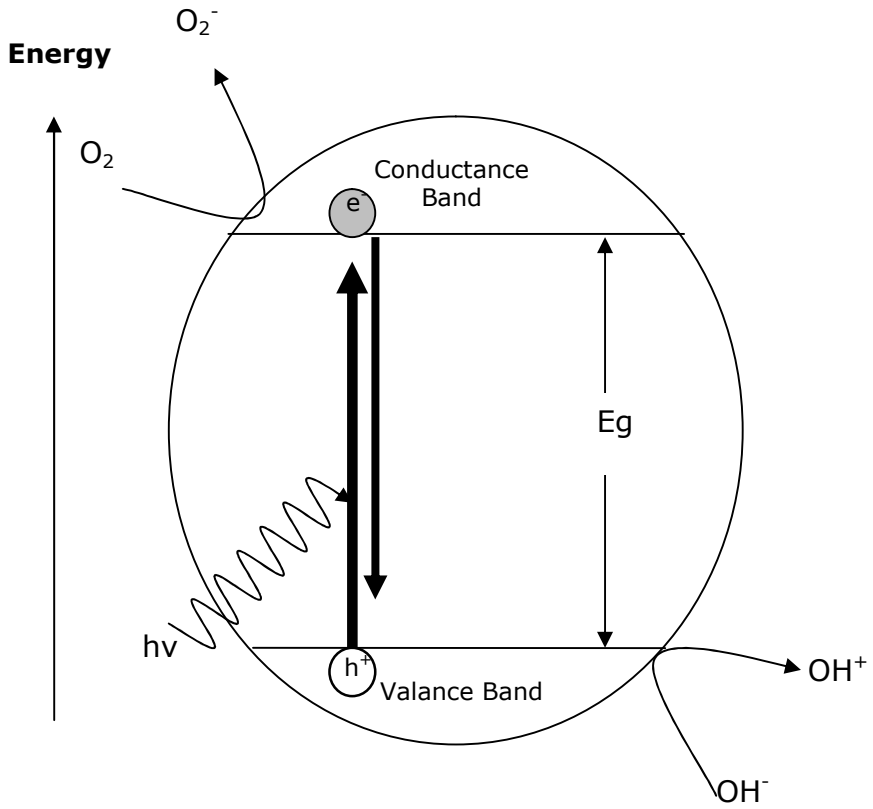

Figure 1.3
An electron is excited by light from the Valance band to the conductance band if enough energy is applied to bridge the energy gap (Eg), where it is capable of reducing species (usually oxygen in air). Once excited by absorbing a photon hv there remains a positive charge in valance band capable of oxidizing compounds and in the case of TiO_2 this oxidizing power is very high.

Once the semiconductor has been excited there are three types of reaction that can occur: the first is a recombination process when the electron and the hole recombine and produce heat as the molecule returns to the unexcited state. The second and third reactions occur when the electron does not return to the hole in the valance band. The hole is left free to accept electrons in an oxidation reaction while the electron in the conductance band can be donated giving rise to a reduction reaction. Depending on the type of semiconductor the oxidation and reduction potentials differ and range from 4.1 to -2.3 volts versus a normal hydrogen electrode (NHE) and are capable of oxidising or reducing a range of compounds. The required energy to promote and electron across the Eg can be calculated in relation to maximum wavelength by the following equation

$\lambda = hc / Eg$. Where λ is the wavelength, h is Plank's constant; c is the speed of light and Eg is the band gap energy between the valance band and the conductance band of the catalyst used.

The choice of catalyst is one of the most important factors and commonly TiO_2 is used as it is not prone to photocorrosion. A process in which the holes left after electron excitation breaks the bonds between the surface atoms of the material and lead to the catalyst dissolving. TiO_2 has been well documented as a photocatalyst for the destruction of pollutants in water as it is chemically passive in a wide pH range and it has a band gap of

3.2 eV and photo excitation occurs with the adsorption of light close to the ultraviolet wavelength (~380 nm). TiO_2 photo-catalysis results in the destruction of compounds by either reduction or oxidation reactions. Research by (Makowski *et al.*, 2001;Robertson *et al.*, 1997;Yamashita *et al.*, 2003;Zhang *et al.*, 2006) have identified that TiO_2 is capable of degrading a wide variety of organic and inorganic compounds as well as bacteria (Zhang *et al.*, 1994).

The mechanisms by which TiO_2 causes the destruction or transformation of compounds under UV irradiation has been extensively investigated, however the photocatalytic degradation mechanisms and intermediates produced when reacting with large and complex molecules is still an area of debate. Although it is thought to that the hydroxyl radicals (OH·) generated in the valance band via oxidation then cause oxidation of any pollutant. As for the electron promoted to the conductance band it is transferred to oxygen to form a superoxide radical anion (O_2^-). Despite gaps in the understanding of the precise mechanisms which occur, the oxidation potential of the hydroxyl radicals (2.8 eV) or the valance band hole (3.1 eV) is considerably greater than that of treatments such as chlorine (1.36 eV), hydrogen peroxide (1.78 eV) or ozone (2.07 eV) (Cornish, 2000).

The potential of using semiconductor photocatalysis to mineralize or remove pollutants has been realized for many years and has therefore received considerable attention in the literature. Significant research has been carried out into understanding the photocatalytic and photooxidizing properties of TiO_2 for many reasons. TiO_2 is capable of absorbing solar radiation, it is non toxic, operates in mild conditions and is quite stable in comparison to other semiconductors with the ability to remain active and is very capable of oxidising a wide range of both toxic and non toxic compounds. Although TiO_2 is able to use solar radiation it is only capable of utilizing about 1% of the solar spectrum. Therefore recent research has focused on modifying the semiconductor TiO_2 so as to improve its ability to utilize a larger proportion of the solar spectrum, making it more viable for drinking water treatment processes including the removal of MCYSTs.

The aims of this thesis can be divided into two key areas. Firstly, studying the influence of inorganic carbon in the growth of cyanobacteria with specific focus on the levels of MCYST produced. As to date it is unclear if elevated inorganic carbon has any influence on the levels of MCYST produced in relation to the biomass and other growth factors such as chlorophyll-a and protein. The levels of MCYSTs have been reported in many ways

mainly against biomass although cell quantity has been more recently used in which a ration of MCYST per cell is quoted. Comparisons have not been reported between the effects on MCYST levels and protein, chlorophyll-a or cell number directly for the same data in order to show if certain factors give a more consistent method for reporting results. Previous research has suggested that the presence of elevated inorganic carbon through the addition of sodium bicarbonate caused significant reductions in the level of MCYSTs produced. Therefore this study aims to determine if the effects observed by (Graham *et al.*, 2001) were as a result of the elevated inorganic carbon or as a result of increased Na^+ on cultures usually occurring in freshwater ecosystems. This study also looked at the effect of increased CO_2 on levels of MCYST and the potential problems which could occur in an atmosphere with a higher CO_2 content than we have presently. Secondly, studies on the destruction of MCYST were also undertaken. Initial studies were carried out to evaluate the natural degradation of a number of MCYST variants and the related nodularin by a range of waters selected for their unique history and prior exposure to MCYSTs. In the event of higher MCYST content in the environment what effect does this have on naturally occurring bacterial populations are they able to degrade such compounds or if catalysts are used how can we

make them more efficient and less expensive to operate in large scale situations?

Further degradation studies were also performed using a novel visible light TiO_2 catalyst to evaluate its potential for further drinking water applications. Visible light activated TiO_2 has the obvious advantages of being able to exploit solar energy, thus greatly reducing the energy required for removing contaminants from potable water.

Chapter 2

Effect of $NaHCO_3$ on MCYST levels produced in cultures of *M.aeruginosa*

2.1 Introduction

The effect of different environmental factors on cyanobacteria and MCYST levels has been investigated from a number of perspectives over the years. A considerable proportion of which has focused on the effect of temperature, light, nutrient availability and seasonal changes, but the true role of these factors still remains elusive. These photosynthetic prokaryotes require three essential components for photosynthesis to occur: carbon dioxide, water and light, to generate energy by the process of photosynthesis. This energy is then used to drive all the major mechanisms required for growth, since the majority of cyanobacteria populate aqueous environments, water is very rarely a limiting factor unlike the availability of inorganic carbon or light. In the presence of increased available inorganic carbon some cyanobacterial cells can increase the production of gas vesicles to alter their buoyancy and access more light for photosynthesis (Klemer *et al.*, 1996). This in turn can result in an increase in growth rate when nutrients such as nitrogen and phosphorus are not limited, high levels of available nutrients can also have a similar effect on cell buoyancy if inorganic carbon or light are limiting optimal growth (Klemer *et al.*, 1996).

The majority of cyanobacteria have been found to proliferate in conditions where the pH is between 7.5 and 11 and are usually

absent from habitats with pH values below 4-5 (Brock, 1973). Although there is no satisfactory understanding of the molecular mechanisms favouring this preference for alkaline environments it should be noted that higher pH favours the formation of bicarbonate and often photosynthesizers are limited by the availability of inorganic carbon (Blanco-Rivero *et al.*, 2005). Krulwich (1995) identified that a wide range of organisms are able to regulate their internal pH through the use of Na^+/H^+ and K^+/H^+ antiporters when external pH is unfavourable. The presence of Na^+ has also been identified (Krulwich, 1995) as essential for the uptake of many inorganic nutrients (inorganic carbon, nitrate and phosphate) but the effect of Na^+ on MCYST levels has to date not been fully investigated.

This Chapter aims to further investigate the findings of (Graham *et al.*, 2001) in which a reduction in MCYST levels was observed, when cultures were grown in the presence of elevated inorganic carbon after the addition of sodium bicarbonate ($NaHCO_3$). The initial studies investigated the effect of different carbonates on MCYST levels in cultures of *M.aeruginosa* PCC7820. It also presents studies which were carried out to determine if the affect on MCYST levels was a direct result of the elevated inorganic carbon or as a result of increasing the levels of Na^+ ions.

2.2 Methods

2.2.1 Growth of Cyanobacteria

The cyanobacteria *Microcystis aeruginosa* PCC7820 (Pasteur Culture Collection, Paris, France) was grown using BG-II medium with nitrate levels modified (Stanier *et al*., 1971) to 8.8 mM (Table 2.1). All cultures were maintained on a regular basis, with sub culturing every 6 weeks into 250 ml conical flasks containing 100 ml of BG-II to give a final culture volume of ~120 ml. The cultures were grown in a temperature controlled room at 25°C with continuous illumination from cool white fluorescent tubes (36 W) 20 µmol s^{-1} m^{-2} (Li-Cor intelligent light meter Li-250).

Table 2.1
Composition of BG-11 plus nitrates growth medium (Stanier *et al.*, 1971)

$NaNO_3$	0.750gl^{-1}
K_2HPO_4	0.040gl^{-1}
$MgSO_4.7H_2O$	0.075gl^{-1}
Na_2CO_3	0.020gl^{-1}
$CaCl_2.2H_2O$	0.036gl^{-1}
EDTA	0.001gl^{-1}
$FeSO_4.7H_2O$	0.006gl^{-1}
Citric Acid	0.006gl^{-1}
Trace element Solution *	1 ml l^{-1}

* Trace element solution	
H_3BO_3	2.680gl^{-1}
$MnCl_2.H_2O$	1.810gl^{-1}
$ZnSO_4.7H_2O$	0.222gl^{-1}
$Na_2MoO_4.2H_2O$	0.390gl^{-1}
Cu $(NO_3)_2.6H_2O$	0.079gl^{-1}
Co $(NO_3)_2.6H_2O$	0.049gl^{-1}

2.2.2 Static culture bioassay conditions

BG-II medium was prepared (Table 2.1) and the pH was adjusted to 7.6 using 0.1 M NaOH / HCl prior to filter sterilizing through a 0.22 µm GP express membrane Millipore filter. The flasks (containing 50 ml of BG-II medium) were inoculated with 2 ml of *M. aeruginosa* culture then grown in water baths at 25°C under continuous illumination from cool white fluorescent tubes (36 W) delivering 30 µmol s^{-1} m^{-2}. Four replicates were run for each condition and the whole culture was removed for sampling at the required time points.

2.2.3 *M. aeruginosa* strain PCC7820 growth curve

Analysis of M.aeruginosa PCC7820 rate of growth was carried out, to determine the time required for cultures post sub-culturing to reach mid log phase. This was to standardize the

starting conditions between experiments and eliminate the possibility of cultures starting from different stages in their natural growth cycle. In order to do this a stock culture of M.aeruginosa PCC7820 was sub-cultured into four 250 ml conical flasks containing 100 ml of BG-II medium to give a final volume of 120 ml. They were then left to grow as detailed above (section 2.2.1) at 25°C under continuous illumination of 20 µmol s^{-1} m^{-2}. Samples were taken daily with chlorophyll-a analysis carried out until the culture reached stationary phase and the results plotted in a graph.

2.2.4 Intracellular processing for MCYST analysis

The amount of intracellular MCYST was calculated by removing a 1 ml aliquot of each sample and placing it into a 1.5 ml micro centrifuge tube. This was centrifuged at 13000 x g for 10 min (Centrifuge 5410, Eppendorf) then 800 µl of supernatant was removed and the sample frozen (overnight). The samples were brought to room temperature on the open bench, then vortexed to re suspend the cells, to which 800 µl of 100 % methanol was added and the samples vortexed until the cells were fully dispersed in solution. These were left to stand for 1 hour before centrifuging for 10 minutes at 13000 x g, after centrifuging 100 µl was taken for analysis by HPLC section 2.2.6.

2.2.5 Procedure for extracellular MCYST analysis

Extracellular toxins form each sample were measured by collecting the supernatant when removing the biomass as explained in section 2.2.15 from (15 ml) of culture, this was then freeze dried (Edwards Modulyo freeze drier fitted with an Edwards RV 5 pump). The freeze dried sample was resuspended in 2 x 0.75 ml of 100% methanol and transferred into a 1.5 ml micro centrifuge tube which was centrifuged for 5 minutes at 13000 x g. A 100 μl sample was removed and analyzed by HPLC using the conditions stated in section 2.2.6.

2.2.6 HPLC analysis of MCYST

Analysis of MCYST variant MCYST-LR, -LY, -LW, -LF and nodularin was performed using the method of (Lawton *et al.*, 1994). High performance liquid chromatography (HPLC) was carried out using a Waters system consisting of a solvent-delivery system model 600E coupled to an autosampler model 717 WISP and detection by photodiode-array (PDA) model 996. Separation of cyanotoxins was carried out using a Waters symmetry C_{18} (250 x 4.6 mm, packing size 5 μm) and chromatography manager software (Waters Millennium[32]). Mobile phase A: Milli-Q water with 0.05 % trifluoroacetic acid

(TFA) and mobile phase B: acetonitrile with 0.05 % TFA. The mobile phases were pumped at a flow rate of 1 ml / min using the gradient shown in (Table 2.2), with helium sparging at 30 ml / min to degas the mobile phase.

Table 2.2
Linear gradient conditions at 1 ml / min for HPLC analysis of MCYST and Nodularin using Solvents A = Water with 0.05% TFA and B = Acetonitrile with 0.05% TFA.

	Time / min						
	0	10	40	42	44	46	55
Solvent A %	70	65	30	0	0	70	70
Solvent B %	30	35	70	100	100	30	30

The column was maintained at 40 °C, standards were injected at 25 μl to give 1 μg column loading and 50 μl injections were used for all samples. The detector was set to acquire data from 200 to 300 nm with resolution of 1.2 nm and the chromatograms monitored at 238 nm. In each run a diluent and MCYST standard were run prior to sample analysis in order to check the system performance. When processing the data the spectra for each peak, is analyzed against the spectral library of known standards in order to determine each peak. If the sample spectrum is similar to that of a standard in the spectral library a match angle is assigned by the Millenium[32] software, the lower this match

angle the better the spectral match. Retention time of known MCYST was also used to confirm identity of peaks.

2.2.7 HPLC analysis limits of detection

The limit of detection in the HPLC-PDA was determined using the method of (Lawton *et al.*, 1994). Purified microcystin-LR was supplied by Dr L.Lawton (The Robert Gordon University, Aberdeen). Different concentrations of MCYST-LR were prepared using a calibrated pipette and 50 μl of each solution was injected. Each solution gave a different column loading ranging from 1 ng to 20 μg, to determine the detectable limit at which acceptable relative standard deviation (RSD) could be achieved. An injector test of the auto sampler was also performed with a solution of MCYST-LR 0.2 μg/ml, six repeat injections of 5, 25, 50, 75 and 100 μl was performed. The RSD of all six injections at each volume must be ≤2.0 %, the Y intercept must be ≥0.999 for the range of injection volumes, and failure to meet the stated criteria shall be explained.

2.2.8 HPLC analysis limits of quantification

Limit of quantification was performed by HPLC analysis using purified MCYST-LR, this was prepared by taking 10 vials of MCYST standard (section 2.2.6) and concentrating the contents

in 2 ml of solvent (80% (aq) methanol). The solvent was added using calibrated pipettes to a pre-weighed glass vial (4 ml) and dried on a dry block DB.3 (Techne) at 45°C and sample concentrator (Techne) under a continuous stream of nitrogen gas until fully dry then weight recorded. The final weight was subtracted from the original weight to calculate the exact amount of MCYST-LR present; this was then suspended in a solution of 80% methanol 20% water. The stock solution was diluted in a series of twelve dilution steps, giving concentrations ranging from 0.02 ng / μl up to 0.4 μg / μl; these were analyzed in triplicate using HPLC Section 2.2.6.

2.2.9 Effect of carbonates on growth and MCYST levels in cultures of *Microcystis aeruginosa* strain PCC7820

BG-II medium was prepared as per Section 2.2.1 this was then separated into four (200 ml) aliquots, to three of the aliquots different bicarbonate salts were added to give a final concentration of 20 mM of sodium bicarbonate, potassium bicarbonate and ammonium bicarbonate. The fourth aliquot was left unaltered and used to prepare experimental controls, the pH of each solution was then altered using 0.1 M hydrochloric acid or 0.1 M sodium hydroxide to give each solution a pH of 7.6 (±0.02). These solutions were then filter sterilized through a

0.22 µm GP express membrane filter, each solution was divided into 25 ml aliquots and added to sterile 50 ml conical flasks (four replicates were prepared for each condition). The cultures were then all inoculated with 2 ml of a dense *M.aeruginosa* PCC7820 culture and then grown in a water bath at 25 ºC under continuous illumination. The cultures were then tested after 6 weeks of growth to determine the levels of intra- and extra-cellular MCYSTs. Due to poor survival of cultures grown in the presence of potassium and ammonium bicarbonate the experiment was terminated early and repeated later at lower concentration of bicarbonate salts (10 mM).

2.2.10 Effect of increased sodium ions on growth and MCYST levels in cultures of *M .aeruginosa* strain PCC7820

As a result of the effects observed during the growth of *M.aeruginosa* PCC7820 in the presence of sodium bicarbonate the effect of increased Na^+ ions was also tested. This was to determine if changes in growth and MCYST levels were as a result of increases in the available inorganic carbon or as a result of increased Na^+ ions. Therefore BG-II medium was prepared as per section 2.2.1 then divided into (200 ml) aliquots prior to the addition of NaCl to give the equivalent level of Na^+ ions as would be present in conditions where $NaHCO_3$ had been

added. Since both NaCl and $NaHCO_3$ contain one Na^+ then if the molarity of a solution is the same then the number of Na^+ ions should also be the same. In addition to having two separate solutions an additional solution of NaCl and $NaHCO_3$ was prepared in which both were added to give a concentration of 10 mM for each and a final concentration of Na^+ ions equivalent to that of 20 mM. The solutions were then filter sterilized, each solution was divided into 25 ml aliquots and added to sterile 50 ml conical flasks (four replicates were prepared for each condition). The cultures were then inoculated with 2 ml of a dense *M.aeruginosa* PCC7820 culture and then grown in a water bath at 25 ºC under continuous illumination. Sampling was carried out after 5 weeks of growth and the levels of intra / extracellular MCYST analyzed by HPLC (Section 2.2.4-2.2.6).

2.2.11 The effect of increasing concentrations of sodium chloride on the growth of *M. aeruginosa* strain PCC7820 and MCYST levels

A variety of concentrations of NaCl were prepared in BG-II medium by first preparing 3 L of BG-II medium as per section 2.2.1 then thirteen 200 ml aliquots were removed, at this stage one aliquot was set aside for the preparation of controls. The remaining aliquots were then split into two groups of six, to the first group of six NaCl was added to give a range of

concentrations 10, 20, 30, 40, 50 and 75 mM. The four remaining aliquots in the second group were then modified with $NaHCO_3$ to give a range of concentrations 10, 20, 30, 40, 50 and 75 mM. Then all aliquots were filter sterilized before being divided into 25 ml aliquots and put into 50 ml conical flasks. The cultures were then all inoculated with 2 ml of a dense *M.aeruginosa* PCC7820 culture and then grown in a water bath at 25 ºC under continuous illumination. Sampling was carried out after 5 weeks of growth and the levels of intra / extracellular MCYST analyzed by HPLC (Section 2.2.4-2.2.6).

2.2.12 Effect of increased Na^+ ions on growth and MCYST levels in BG-II media in the presence of NaCl, $NaHCO_3$ and a combination of both.

As a result of the concentrations above 40 mM $NaHCO_3$ being detrimental to the growth of *M.aeruginosa* PCC7820 in the experiment above, it was decided to carry out a similar experiment but with a maximum concentration of 40 mM for NaCl, $NaHCO_3$ and added together. An additional test was also carried out to test what effect a combination of both NaCl and $NaHCO_3$ being present at the same time on the growth of *M.aeruginosa* PCC7820. BG-II media was prepared as per section 2.2.1 and divided into (200 ml) aliquots to which the following concentration ranges (10, 20, 30 and 40 mM) were

prepared for both NaCl and $NaHCO_3$ separately. Then one aliquot was prepared with 10 mM of NaCl and 10 mM $NaHCO_3$ to give a solution with an elevated inorganic carbon level and a doubly high concentration of Na^+ ions than has previously been used. The aliquots were then filter sterilized before being divided into 25 ml aliquots and put into 50 ml conical flasks. Each culture flask was then inoculated with 2 ml of a dense *M.aeruginosa* PCC7820 culture and then grown in a water bath at 25 ºC under continuous illumination. Sampling was carried out after 5 weeks of growth and the levels of intra / extracellular MCYST analyzed by HPLC (Section 2.2.4-2.2.6).

2.2.13 Standardization of static culture bioassay starting conditions

BG-II media was prepared (Table 2.1) and the pH was adjusted to 7.6 using 0.1 M NaOH / HCl prior to filter sterilization. The media was then inoculated with log phase cells to a density of ~1.5 million cells / ml, the freshly inoculated media was then divided into 20 ml aliquots and put into sterile 50 ml conical flasks. The cultures were then grown in water baths at 25°C under continuous illumination from cool white fluorescent tubes (36 W) delivering 20 µmol s^{-1} m-2. Four replicates were run for

each condition and the whole culture was removed for biomass, intra- and extra-cellular analysis at the required time points.

2.2.14 Effect on growth and MCYST levels of buffering BG-II medium

BG-II medium was prepared as per (Table 2.1), this was then separated into three (600 ml) aliquots, TES (N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid, Sigma, Aldrich) buffer or HEPES (N-cyclohexyl-2-aminoethane sulfonic acid, Sigma, Aldrich) buffer was added to two of the aliquots to produce BG-II with 20 mM buffer. Sodium bicarbonate was then added to one of the aliquots to produce a solution of 20 mM sodium bicarbonate. The pH of each solution was altered using 0.1 M hydrochloric acid or 0.1 M sodium hydroxide to give a solution at pH 7.6 (± 0.02), the three aliquots of media were then filter sterilized. The media was then inoculated with log phase cells to a density of ~1.5 million cells ml^{-1}, the freshly inoculated media was then divided into 20 ml aliquots and put into sterile 50 ml conical flasks. The cultures were grown in three conditions: control (BG-II only), BG-II with 20 mM HEPES and BG-II with 20 mM HEPES + 20 mM sodium bicarbonate, each test was carried out in quadruplicate. The cultures were grown under the conditions stated in section 2.2.2 then at the

required time points: pH, biomass, cell counts, chlorophyll a, protein, intra and extracellular analysis carried out.

2.2.15 Biomass determination

To determine the cell biomass for each condition, 15 ml of sample was removed and filtered under vacuum through a pre-weighed GF/C (Watman Ltd., Kent, England) filter (1.2 µm pore size). The GF/C filter was put into a Petri dish and dried at 70 °C in an oven overnight. The filters were removed and left to reach ambient temperature before weighing; the initial filter weight was then subtracted from the final weight to give the weight of biomass per 15 ml of sample. The biomass was then converted to mg / ml of culture and used to calculate toxin amounts relative to the cell biomass.

2.2.16 Chlorophyll-a

The amount of Chlorophyll-a present within the controls and sample cultures was measured by removing 1 ml of culture and putting it into a 1.5 ml microcentrifuge tube. These were then centrifuged for 10 min at 13000 x g, after which 800 µl of supernatant was removed. Then 800 µl of 100 % methanol was added, vortexed and left for 1 hour to extract the chlorophyll-a from the cells. The samples were then centrifuged for 10 min

and the absorbance of the solution taken at 665 nm. The results were then converted into µg / ml chlorophyll-a (Murphy *et al.*, 2005).

$$\text{Chlorophyll a } (\mu g / ml) = \frac{13.0 \times A \times \nu}{d \times V}$$

Where A = absorption at 665nm
ν = solvent in ml
V = initial filtered sample volume in L
d = cell path length in cm µg / ml

2.2.17 Cell counts

Cell counts were carried out by removing 1 ml of sample from which 100 µl was taken and diluted in 5 ml of cell counting Sysmex diluent. The sample was then counted using a Sysmex F-520P a semi-automated sample measurement unit coupled to a particle size distribution analyzer Sysmex PDA 500AD (Malvern Instruments LtD. Worcestershire),in order to gain not only the number of cells present but also the size distribution. The F-520P removes 500 µl of diluted sample (cells) through a 100 µm orifice in the transducer and the particle sizes are detected. A distribution graph of the particle sizes is displayed with cell counts / ml and the proportion of over counting which should be ≤20 cells / ml. Any samples with over counts >20 cells / ml had the analysis repeated to verify the results.

2.2.18 Protein analysis

The level of intracellular protein in samples was analyzed using the Lowery assay method, which is a reliable method for protein quantification with little variation among different proteins. The sensitivity of this method is from 5 to 100 µg / ml (Lowry *et al.*, 1951). A 1 ml sample of culture was removed and centrifuged at 13000 x g (Centrifuge 5410, Eppendorf) then 800 µl of supernatant was removed and the sample frozen (overnight). The samples were removed from the freezer and then freeze dried, before being reconstituted in 500 µl of Milli- Q water. They were then vortexed for ~1 min and left to stand for 1 hour before 10 min of centrifugation (13000 x g). Standards were then prepared using 10 µg of bovine serum albumin in 10 ml of distilled water to give a stock solution of 1 µg / ml protein.

This was used to prepare a series of standard dilutions to create a calibration graph and quantify the amount of protein present within each sample. The following solutions were prepared for the Lowery analysis.

- Solution A:- Cupric sulphate pentahydrate with Tri sodium citrate added to 1 L of distilled water.
- Solution B:- Sodium carbonate and sodium hydroxide added to 1 L of distilled water.
- Solution C:- Mixture of solutions A and B in a ratio of 1:50.

- Solution D:- Folin-Ciocalteu phenol reagent diluted at a ratio of 50:50 with distilled water.

The assay was carried out by taking 125 µl of sample or standard and placing in a 1.5 ml micro centrifuge tube to which 625 µl of solution C was added. The solutions were mixed by vortexing for a few seconds before allowing too stand at room temp for 5-10 min, then 63 µl of solution D was added and the sample vortexed again. After which the sample was placed in the dark for 20-30 min to develop before their absorbances were read at 650 nm. The standard curve was then used to quantify the amount of protein in each sample if the R^2 value was $\geq$0.999.

2.2.19 Standardization of experimental inoculation

BG-II media was prepared as per Table 2.1, this was then separated into four (600 ml) aliquots, and to two of the aliquots sodium bicarbonate was added to produce solutions of 10 mM, 20 mM and 30 mM sodium bicarbonate. The pH of each solution was altered using 1 M hydrochloric acid or 1 M sodium hydroxide to give a solution at pH 7.6 (± 0.02), the four aliquots of media were then filter sterilized. The media was then inoculated with log phase cells to a density of ~1.5 million cells / ml, the freshly inoculated media was then divided into 20 ml aliquots and put

into sterile 50 ml conical flasks. The cultures were grown in four conditions control (BG-II only); BG-II with 10 mM sodium bicarbonate, BG-II with 20 mM sodium bicarbonate and BG-II with 30 mM sodium bicarbonate all had four replicates. They were grown under the conditions stated in section 2.2.2 and analyzed at the required time points.

2.3 Results and discussion

2.3.1 Effect of different bicarbonates on growth in cultures of *M. aeruginosa* strain PCC7820

Previously (Graham *et al.*, 2001) reported that the presence of elevated inorganic carbon through the addition of $NaHCO_3$ had profound effects on the level of MCYST after 6 weeks of growth. Their research however did not determine if the reductions in MCYST levels were as a result of the elevated inorganic carbon or sodium ions. Therefore initial investigations to determine which of these components were causing this effect were investigated through the use of different types of bicarbonates. Using this approach the levels of inorganic carbon were increased without affecting the levels of Na^+ ions present. The experiments were carried out (Section 2.2.8) using three types of bicarbonate, $NaHCO_3$, $KHCO_3$, and NH_3HCO_3. Only the controls and $NaHCO_3$ cultures grew, in both the $KHCO_3$, and NH_3HCO_3 cell death occurred. The cause of cell death in the $KHCO_3$ supplemented medium may be due to evidence presented by (Parker *et al.*, 1997) where they report that potassium ions strongly inhibit growth of *Microcystis*. The presence of increased ammonium in media is also well documented as inhibitory to many micro-organisms.

2.3.2 Effect of elevated Na^+ ions on culture growth and on the levels of MCYST produced.

The effect of sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) on intracellular levels of MCYST in cultures of *M. aeruginosa* species PCC7820 after five weeks of growth were investigated (Table 2.3) and compared to controls. The presence of either 10 mM NaCl or 10 mM $NaHCO_3$ increased biomass by 16% and 54%, respectively compared to control cultures and with an SD of 0.07 was more consistent than the controls with an SD of 0.22. Using the ANOVA one way test a p value of 0.00998 ($p<0.01$) and statistically indicated a direct relationship between NaCl and growth in *M.aeruginosa* PCC7820. A significant difference ($p<0.05$) between the control and the NaCl 10 mM condition but not a significant difference between the control and $NaHCO_3$ at 10 mM was observed. This was different from the research by (Liu, 2006) who found an indirect relationship between salinity and growth in cultures of *M.aeruginosa* UTEX 2385. However research (Parker *et al.*, 1997) using sodium salts also found a significant difference when *microcystis spp.* were grown in the presence of 20 mM $NaHCO_3$. Likewise they also observed that NaCl enhanced growth when applied at 30 mM, while they did not directly compare the effects of $NaHCO_3$ and NaCl it was observed that enhanced growth was only apparent in the NaCl treated cultures

after 10 days growth. In the present study increases in the biomass (mg) of dry weights were observed in both $NaHCO_3$ and NaCl although readings were only made after 5 weeks of treatment.

Condition	mg of biomass / ml of culture	
	Mean	± S.D
Control	0.84	0.22
NaCl 10 mM	0.97	0.03
$NaHCO_3$ 10 mM	1.29	0.07

Table 2.3
Biomass weights for cultures of *M.aeruginosa* PCC7820 grown in the presence of $NaHCO_3$ and NaCl at concentrations ranging from 10 mM.

The yield of intracellular MCYST-LR when calculated in relation to the dry weights of biomass (Figure 2.1) was found to be enhanced (increased by 22%) in the NaCl treated cells. However, such an increase was not observed in the cultures treated with $NaHCO_3$, but these exhibited a similar trend to previous results reported by (Graham *et al.*, 2001) where the presence of 10 mM $NaHCO_3$ appeared to suppress MCYST yield and a decrease of over 20% was reported, although in this experiment the decrease was much smaller at a 7% decrease. In order to fully determine if MCYST levels were being

suppressed through the addition of $NaHCO_3$ it was also vital to examine the extracellular proportion as any intracellular reduction may have been as a result of export or cellular leakage.

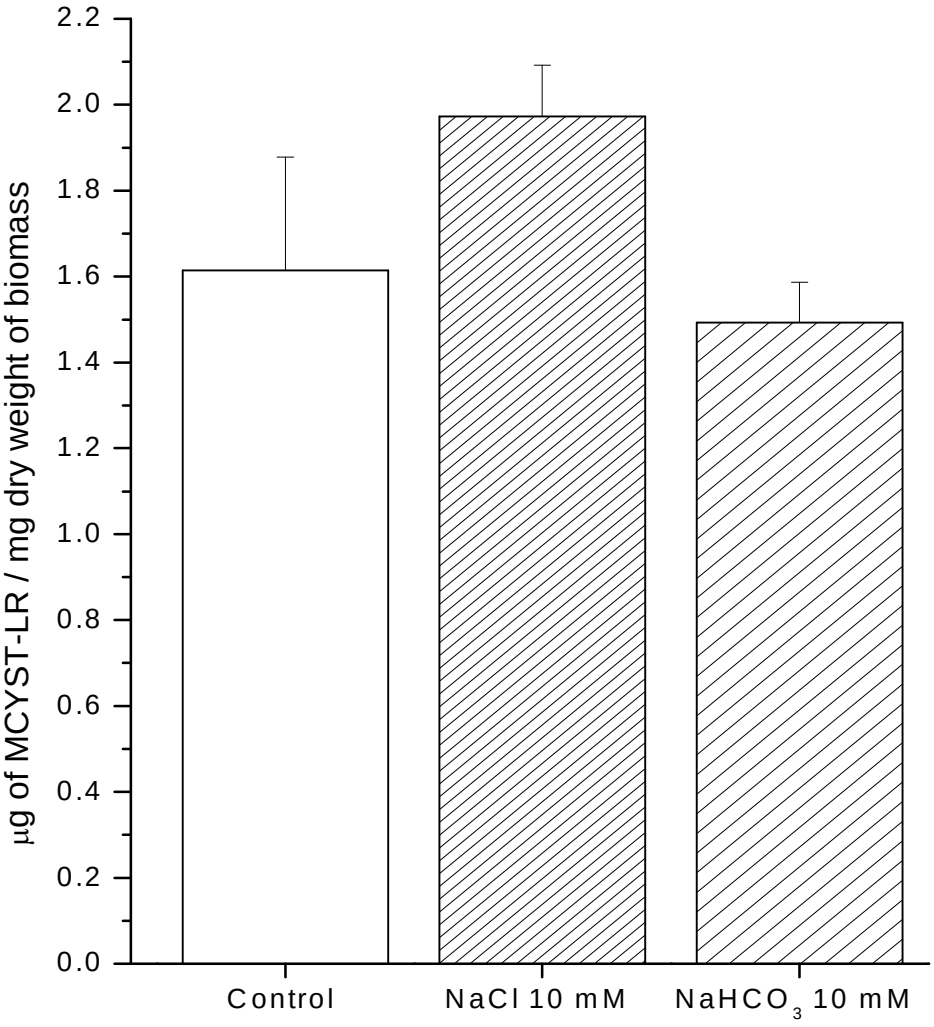

Figure 2.1
Effect on intracellular MCYST-LR quantity in *M. aeruginosa* PCC7820 when increased Na^+ ions are present by the addition of NaCl, or $NaHCO_3$ both at 10 mM ▨.

Although Na^+ is an essential ion for most cyanobacteria especially during growth in elevated pH environments it is an essential component in the uptake of several nutrients (inorganic carbon, nitrate, phosphate) and for photosynthetic electron transport (Blanco-Rivero *et al.*, 2005). However high concentrations of Na^+ can cause cell stress and many species of freshwater cyanobacteria are unable to grow in high saline environments. MCYST are mainly found intracellularly and are not usually released into the surrounding media (Jones *et al.*, 1994;Orr *et al.*, 2004) unless cell lysis occurs. However, results by (Orr *et al.*, 2004) observed a significant decrease in intracellular MCYST concentration in the presence of 21.2 g / L (365.5 mM) NaCl and a significant increase in the proportion of extracellular MCYST. In our study (Table 2.4) relatively little extracellular MCYST was detected in the elevated NaCl but the concentration of NaCl was substantially lower than in the study by (Orr *et al.*, 2004). However it was observed that 28% of the total MCYST was detected in the media in cells grown in the presence of $NaHCO_3$. In comparison only 3 and 4% was detected in the media of the control and 10 mM NaCl condition respectively (Table 2.4).

Condition	Intracellular		Extracellular		Total
	%	µg / ml of culture	%	µg / ml of culture	µg / ml of culture
Control	97	1.31	3	0.04	1.35
NaCl 10 mM	96	1.91	4	0.07	1.98
$NaHCO_3$ 10 mM	72	1.91	28	0.73	2.64

Table 2.4
Comparison of the intra and extracellular portions of MCYST-LR in the presence of elevated Na^+ ions with either Cl^- or HCO^-_3 present.

These results would support the idea that the effect on extracellular MCYST levels observed with elevated $NaHCO_3$ was not as a result of increased Na^+. While few other published reports have studied the effect of Na^+ on MCYST more detailed research has focused on the brackish water cyanobacteria *Nodularia* which produces the related toxin nodularin. As might be expected for a brackish organism growth would be lowest when no Na^+ was added and increased at the higher concentrations. Furthermore (Blackburn *et al.*, 1996) observed that as salinity increased toxin concentration decreased, which is in contrast to the findings here where elevated Na^+ appeared to actually increased intra-MCYST levels (Figure 2.1 and table 2.4). Studies using a range of strains of *N. spumigenia* isolated from the Baltic Sea demonstrated a link between Na^+ and toxin levels although the differences were much more profound in one specific strain BY1. The growth of cyanobacteria in increased Na^+ may be related to their ability to adjust respiration and

regulate the intake or efflux of Na^+ to enhance cyclic electron transfer via photosystem-I and organic osmolytic compound production (Moisander *et al.*, 2002). Other researchers (Paerl *et al.*, 1983;Sellner *et al.*, 1988) indicated that high salinities are inhibitory for growth and CO_2 fixation of the non-N_2-fixing, toxic, unicellular MCYST *M. aeruginosa*. Active ion export and expression of stress proteins or other salt acclimation mechanisms present in cyanobacteria can result in reduced cell growth. These metabolic responses to salt stress usually result in reduce growth rates and complete growth inhibition can occur in some species. Research in eukaryotic algae and cyanobacteria has shown salt stress to be a major cause of increased light sensitivity and even photoinhibition (Moisander *et al.*, 2002). Verspargen *et al.,* (2006) over a four year study into seasonal blooms found significant growth inhibition, as salinity increased and almost complete inhibition at concentrations of 12 g / L (205 mM) (Verspargen *et al.*, 2006). Lehtimäki *et al,.* (1994) reported that intra-cellular toxicity was not affected by an increase in salinity between 51 to 188 mM, but instead suggested that the levels of extracellular toxin were increased. This type of relationship was however not observed in this study as increased levels of extracellular MCYST were not detected in any of the cultures even when subjected to concentrations of 75 mM NaCl (Table 2.6). Also in cultures grown in the presence of

both NaCl and $NaHCO_3$ together as high as (30 mM) no significant change in extracellular MCYST was observed see Table 2.12 in results Section of 2.3.5 later.

2.3.3 Effect of different concentrations of NaCl and $NaHCO_3$ on MCYST levels

As a result of the observations from the previous experiment a more detailed investigation into how NaCl and $NaHCO_3$ affects the levels of MCYST in cultures of *M. aeruginosa* PCC7820 over a period of 5 weeks was studied. NaCl and $NaHCO_3$ were tested at concentrations of 10 mM to 75 mM, with biomass and HPLC analysis of intra and extracellular quantities determined for MCYST-LR. The effect on biomass (Table 2.5) indicates that growth of *M. aeruginosa* PCC7820 is halted by the presence of 40 mM $NaHCO_3$ and greater concentrations. Also as the concentration increases from 10 - 30 mM a small biomass decrease was observed in comparison to the controls. Increasing levels of NaCl however did not affect the biomass in the same way as observed in the $NaHCO_3$ cultures. The biomass from NaCl (10 - 50 mM) cultures was either higher or equivalent to the levels observed in the controls, a reduction in biomass was only observed at the highest concentration of 75 mM.

Condition	Biomass		Intracellular MCYST-LR	
	Mean mg / ml of culture	±S.D	µg / mg of biomass	%*
Control	1.25	0.29	2.80	-
$NaHCO_3$ 10 mM	1.15	0.08	2.07	-26
$NaHCO_3$ 20 mM	0.99	0.06	1.12	-60
$NaHCO_3$ 30 mM	1.01	0.09	0.72	-74
$NaHCO_3$ 40 mM	N/G	N/G	N/G	N/G
$NaHCO_3$ 50 mM	N/G	N/G	N/G	N/G
$NaHCO_3$ 75 mM	N/G	N/G	N/G	N/G
NaCl 10 mM	1.33	0.14	2.08	-26
NaCl 20 mM	1.38	0.05	1.58	-43
NaCl 30 mM	1.13	0.12	1.70	-39
NaCl 40 mM	1.30	0.15	1.21	-57
NaCl 50 mM	1.10	0.18	1.42	-49
NaCl 75 mM	0.89	0.14	0.39	-86

Table 2.5
Biomass weights and MCYST-LR levels for cultures of *M.aeruginosa* PCC7820 grown for 5 weeks in the presence of $NaHCO_3$ and NaCl at concentrations ranging from 10 mM to 75 mM (N/G = No Growth, ±S.D = standard deviation, n = 4).* Percent variation when compared to control.

Analysis of intracellular MCYST-LR in the elevated $NaHCO_3$ indicated a decreasing trend when the MCYST levels were expressed in relation to the biomass weights (Table 2.5 and Figure 2.2). Initially there was a 26% decrease in MCYST-LR cultures treated with 10 mM $NaHCO_3$, 60% decrease for 20 mM and 74% for those treated with 30 mM. While in the cultures of

NaCl the level of MCYST detected were also reduced with the concentration at 10 mM conditions caused a 26% reduction and 20-50 mM conditions had reductions within a range of 40 - 57%. The presence of 75 mM NaCl caused the highest reduction with some 86% reduction occurring, probably due to poor growth and cellular stress caused by the Na^+ ions.

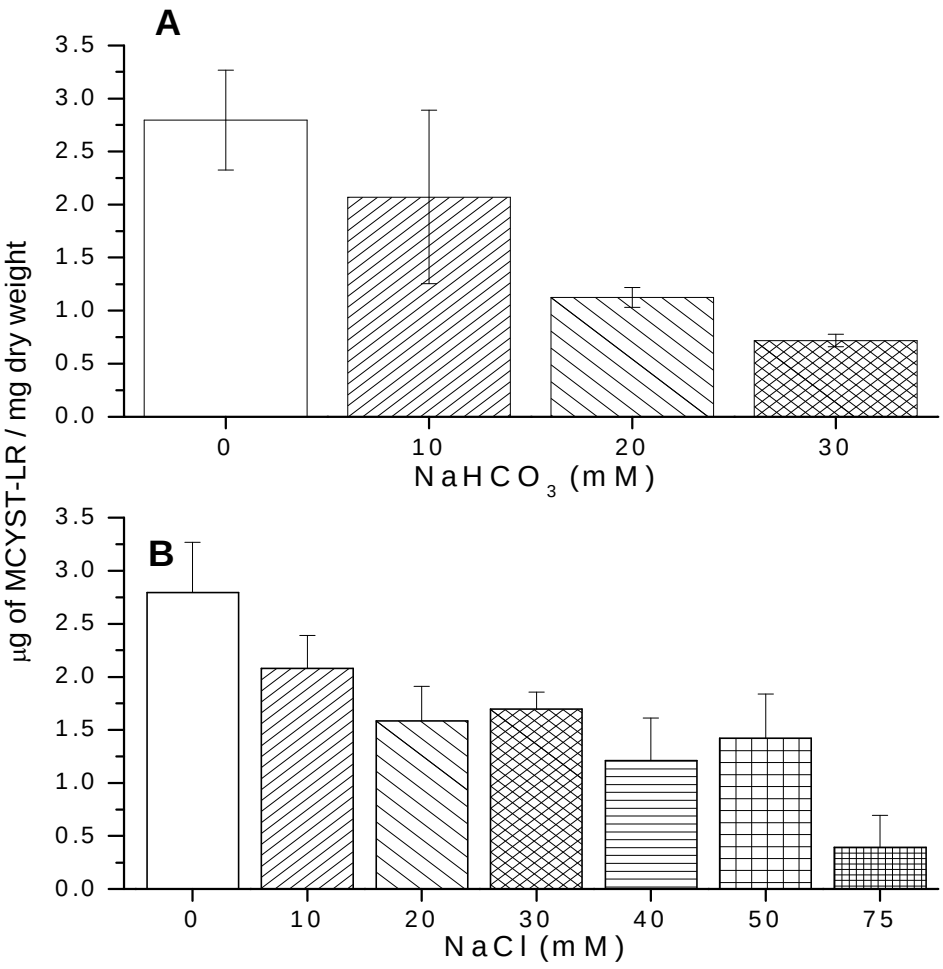

Figure 2.2
The effect on cultures of *M.aeruginosa* PCC7820 MCYST-LR levels when subjected to increasing concentrations (A) $NaHCO_3$ and (B) NaCl.

MCYST levels reported (Table 2.6) show that the levels of MCYST-LR as the concentrations of $NaHCO_3$ and NaCl increase the percentage of extracellular MCYST detected decreases, with the lowest extracellular levels found at the highest concentrations. It was also apparent when comparing the total MCYST µg / ml, that the presence of $NaHCO_3$ and NaCl caused a reduction in the level of MCYST. It is also apparent that the total MCYST-LR (µg / ml) in the controls was higher than that observed in either of the cultures supplemented with NaCl or $NaHCO_3$. This experiment was prior to standardization of inocula and although cultures were all inoculated from a batch culture the inoculation was by volume of culture and not on cell density. Also the age of the culture had not been set to avoid using cells out with the mid log phase to reduce lag time after inoculation and reduce the possibility of cells being at different stages in the cell cycle. It is well documented that stationary phase cells contain higher levels of MCYST than those in lag or log phase, since less energy is being expended on growth and many cells are more fragile with a tendency to leak easier.

Condition	Intracellular		Extracellular		Total
	%	µg / ml	%	µg / ml	µg / ml
Control	60	3.41	40	2.27	5.68
$NaHCO_3$ 10 mM	48	2.39	52	2.60	4.99
$NaHCO_3$ 20 mM	71	1.11	29	0.45	1.56
$NaHCO_3$ 30 mM	86	0.73	14	0.12	0.85
$NaHCO_3$ 40 mM	N/G	N/G	N/G	N/G	N/G
$NaHCO_3$ 50 mM	N/G	N/G	N/G	N/G	N/G
$NaHCO_3$ 75 mM	N/G	N/G	N/G	N/G	N/G
NaCl 10 mM	57	2.74	43	2.06	4.80
NaCl 20 mM	56	2.17	44	1.74	3.91
NaCl 30 mM	78	1.90	22	0.54	2.44
NaCl 40 mM	92	1.60	8	0.14	1.75
NaCl 50 mM	96	1.52	4	0.06	1.58
NaCl 75 mM	96	0.36	N/D	0.01	0.37

Table 2.6
Percent of intracellular and extracellular MCYST-LR levels for cultures of *M.aeruginosa* PCC7820 grown for 5 weeks in the presence of $NaHCO_3$ and NaCl at concentrations ranging from 10 mM to 75 mM (n = 4, % mean in parenthesis, N/D = Not Detected).

The pH of the media after 5 weeks of growth had increased in both the $NaHCO_3$ and NaCl conditions, to varying degrees Table 2.7. Generally thought the pH in cultures subjected to different concentrations of $NaHCO_3$ showed an increase in pH from 7.60 to an average of 9.43. After the 5 weeks of growth in the presence of increased NaCl however the final pH was lower than

in the control cultures with an average pH of 8.67, a decrease of 0.53. This may possibly restrict the nature in which MCYST-LR can be exported from within the cell, as the lowest pH was observed in the 75 mM condition which also had the lowest extracellular proportion. However further research is required to investigate this hypothesis, as it may be just a coincidence and the process is actually controlled by other mechanisms.

	pH at start	**pH at End**	**Difference**
Control	7.50	9.21	1.71
$NaHCO_3$ 10mM	7.71	9.36	1.65
$NaHCO_3$ 20mM	7.68	9.41	1.73
$NaHCO_3$ 30mM	7.65	9.47	1.82
$NaHCO_3$ 40mM	7.68	9.48	1.80
NaCl 10mM	7.65	9.02	1.37
NaCl 20mM	7.63	8.93	1.30
NaCl 30mM	7.63	8.84	1.21
NaCl 40mM	7.68	8.67	0.99
NaCl 50mM	7.74	8.63	0.89
NaCl 75mM	7.75	7.98	0.23

Table 2.7
Comparison of the pH at the start and end on the experiment for cultures on *M.aeruginosa* PCC7820 grown in the BG-II media with a range of concentrations of both $NaHCO_3$ and NaCl.

2.3.4 Effect of combining $NaHCO_3$ and NaCl on MCYST levels

As a result of the previous experiments in which the effect of NaCl and $NaHCO_3$ had been tested separately at various concentrations, it was decided to test the effect of combining the two. In an attempt to see what effect increased Na^+ ions would have when both the Cl^- and HCO_3^- were also present at levels previously tested separately. A culture containing a combination of both NaCl (10mM) and $NaHCO_3$ (10mM) was prepared to give a Na^+ ion level equivalent to that of 20 mM. This was compared to a range of individual NaCl and $NaHCO_3$ concentrations (10 - 40 mM) grown under the same conditions. The effect each condition had on the biomass (Table 2.6) shows an increased level of biomass in cultures subjected to both NaCl and $NaHCO_3$ combined in comparison to the control and those tested with either NaCl or $NaHCO_3$ alone.

Condition	Biomass		Intracellular MCYST-LR	
	Mean mg / ml of culture	± S.D	µg / mg of biomass	% *
Control	1.21	0.13	2.16	-
$NaHCO_3$ 10 mM	1.10	0.08	1.91	-11.5
$NaHCO_3$ 20 mM	1.14	0.10	2.31	+7.2
$NaHCO_3$ 30 mM	0.74	0.05	1.64	-24.1
$NaHCO_3$ 40 mM	N/G	N/G	N/G	N/G
NaCl 10 mM	1.08	0.10	2.74	+26.8
NaCl 20 mM	1.02	0.15	2.73	+26.6
NaCl 30 mM	1.06	0.17	2.26	+4.9
NaCl 40 mM	0.96	0.05	1.98	-8.2
NaCl 10 mM + $NaHCO_3$10 mM	1.38	0.08	1.74	-19.5

Table 2.8
Biomass weights for cultures of *M.aeruginosa* PCC7820 cultures tested over a variety of concentrations subjected to both NaCl + $NaHCO_3$, $NaHCO_3$ and NaCl at concentrations ranging from 10 mM to 40mM mM (N/G = No Growth, ±S.D = standard deviation, n = 4).* Percent variation when compared to control.
.

The levels of MCYST were calculated against the dry weight of cells (Figure 2.3) in the presence of $NaHCO_3$ at 10 – 30 mM and the level of MCYST fluctuated but no specific trend was observed. There can be no obvious reason why this is not comparable to the reduction trend observed in the previous experiment although it may highlight the importance of standardizing inoculation conditions as was adopted in later experiments. Also at 40 mM only one of the four replicates grew

and therefore the data was not included. In the presence of NaCl however an increase was observed in the 10 – 20 mM conditions but at a higher level of 30 – 40 mM a gradual decrease was observed. In the presence of both NaCl and $NaHCO_3$ the intracellular level of MCYST was 20% lower than the control level and when compared to the 10 mM levels of NaCl and $NaHCO_3$ separately where there was a 27% increase and an 11% decrease respectively. This suggests that the presence of increased inorganic carbon is the cause of the reduction in intracellular MCYST and not the presence of increased Na^+ ions. As for the levels of extracellular MCYST (Table 2.9) the presence of $NaHCO_3$ at the lowest concentration was identical to the control but as the concentration increased the percentage of extracellular MCYST also increased. A similar trend was not however observed in the presence of NaCl as no extracellular toxin was detected or the levels were below the limits of detection. Interestingly these results demonstrate that in the presence of both NaCl and $NaHCO_3$ it might be expected that higher Na^+ content causes a lower extracellular MCYST level. Instead, over 80% of extracellularly MCYST was detected which can only be as a result of a combined effect because neither of the two 20 mM conditions show this type of behaviour.

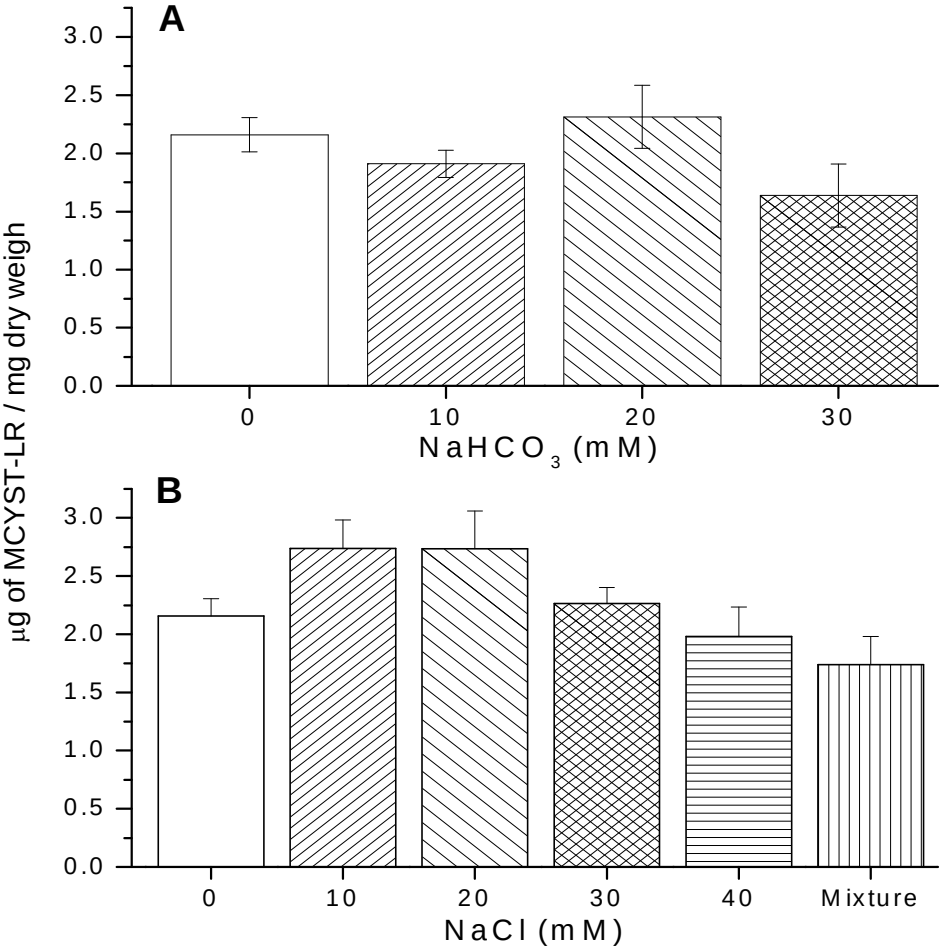

Figure 2.3
The effect on cultures of *M.aeruginosa* PCC7820 MCYST-LR levels when subjected to increasing concentrations (A) $NaHCO_3$ and (B) NaCl and Mixture (combination of both10 mM of NaCl and 10 mM $NaHCO_3$).

Condition	Intracellular		Extracellular		Total
	%	µg / ml	%	µg / ml	µg / ml
Control	77	2.60	23	0.77	3.37
$NaHCO_3$ 10 mM	77	2.10	23	0.63	2.37
$NaHCO_3$ 20 mM	4	2.65	96	0.12	2.77
$NaHCO_3$ 30 mM	100	1.15	N/D	N/D	1.15
$NaHCO_3$ 40 mM	N/G	N/G	N/G	N/G	N/G
NaCl 10 mM	100	2.93	N/D	N/D	2.93
NaCl 20 mM	100	2.74	N/D	N/D	2.74
NaCl 30 mM	100	2.40	N/D	N/D	2.40
NaCl 40 mM	100	1.91	N/D	N/D	1.91
NaCl 10 mM + $NaHCO_3$ 10 mM	16	2.39	84	0.46	2.85

Table 2.9
Percent of intracellular and extracellular toxin for MCYST-LR levels identified in cultures tested over a variety of concentrations although extracellular MCYST-LR was only found in two of the $NaHCO_3$ conditions and in the condition subjected to both NaCl + $NaHCO_3$ ((n = 4, % = %mean in parenthesis, N/D = Not Detected and N/G = No growth).

The pH at the end of the experiment was tested (Table 2.10) and in all the conditions an increase was observed from the starting pH of 7.60, with the NaCl conditions pH being slightly higher than the control at 3.5% higher. A much higher percent increase was observed however in the presence of $NaHCO_3$ and NaCl with $NaHCO_3$ with an increase of 8.7% and 7.0%

respectively suggesting the higher pH may be associated with an increase in extracellular MCYST.

Condition	pH	
	Mean	±S.D
Control	8.55	0.16
$NaHCO_3$ 10mM	9.10	0.02
$NaHCO_3$ 20mM	9.14	0.06
$NaHCO_3$ 30mM	9.45	0.02
$NaHCO_3$ 40mM	9.47	0.00
NaCl 10mM	8.95	0.04
NaCl 20mM	8.90	0.04
NaCl 30mM	8.79	0.04
NaCl 40mM	8.75	0.03
NaCl+$NaHCO_3$ 10mM	9.15	0.07

Table 2.10
Comparison between the experimental conditions pH at the end of the experiment. (±S.D = Standard deviation)

2.3.5 Detailed investigation into the effect of increased Na^+ in the presence of elevated inorganic carbon

As a result of the previous experiment where poor growth was observed in the 40 mM $NaHCO_3$ condition it was decided to reduce the concentrations being tested and increase the range for the condition containing both NaCl and $NaHCO_3$. Three conditions were tested at five different concentrations 2, 5, 10, 20 and 30 mM, their biomass weights were taken (Table 2.11)

with very little difference observed between the three conditions. Only in the presence of $NaHCO_3$ at 20 mM was a large reduction in weight observed but this may be due to poor growth as the 30 mM $NaHCO_3$ condition did not grow as in previous experiments (unexplainable culture death).

Condition	Biomass		Intracellular MCYST-LR	
	Mean mg / ml	± S.D	µg / mg	%*
Control	1.38	0.07	1.31	-
$NaHCO_3$ 2 mM	1.33	0.08	1.42	8.7
$NaHCO_3$ 5 mM	1.35	0.02	1.52	16.4
$NaHCO_3$ 10 mM	1.30	0.07	1.51	15.7
$NaHCO_3$ 20 mM	0.83	0.11	0.84	-35.6
$NaHCO_3$ 30 mM	N/G	N/G	N/G	N/G
NaCl 2 mM	1.31	0.10	1.65	26.3
NaCl 5 mM	1.22	0.25	1.81	38.5
NaCl 10 mM	1.40	0.10	1.57	19.9
NaCl 20 mM	1.37	0.11	1.65	26.6
NaCl 30 mM	1.28	0.02	2.07	58.1
NaCl + $NaHCO_3$ 2 mM	1.30	0.17	1.73	32.2
NaCl + $NaHCO_3$ 5 mM	1.51	0.09	1.34	2.2
NaCl + $NaHCO_3$ 10 mM	1.25	0.11	1.57	20.0
NaCl + $NaHCO_3$ 20 mM	1.21	0.04	1.42	8.5
NaCl + $NaHCO_3$ 30 mM	1.18	0.07	1.64	25.4

Table 2.11
Biomass weights for cultures of *M.aeruginosa* PCC7820 cultures tested over a variety of concentrations subjected to both; NaCl + $NaHCO_3$, $NaHCO_3$ and NaCl at concentrations ranging from 2 mM to 30 mM. (N/G = No Growth, ±S.D = standard deviation, n = 4). * Percent variation when compared to controls.

Intracellular MCYST analysis was carried out (Figure 2.4) with the results calculated as µg of MCYST-LR / mg dry weight and in the presence of increased inorganic carbon through the addition of $NaHCO_3$ there was only a slight increase in the level of MCYST-LR detected after 6 weeks of growth. In the presence of NaCl however the intracellular level of MCYST increased gradually from 2 to 5 mM but a linear increase was not observed for the 10 and 20 mM although they were still higher than the control but the highest increase was observed in the presence of 30 mM NaCl. The main purpose of this experiment however was to determine what effect the presence of NaCl and $NaHCO_3$ together would have on MCYST levels as in the previous experiment there was less intracellular MCYST in comparison to the control. A similar reduction was not observed in this experiment though and increasing the concentration did not follow any specific trend i.e. increasing or decreasing as the concentration increases.

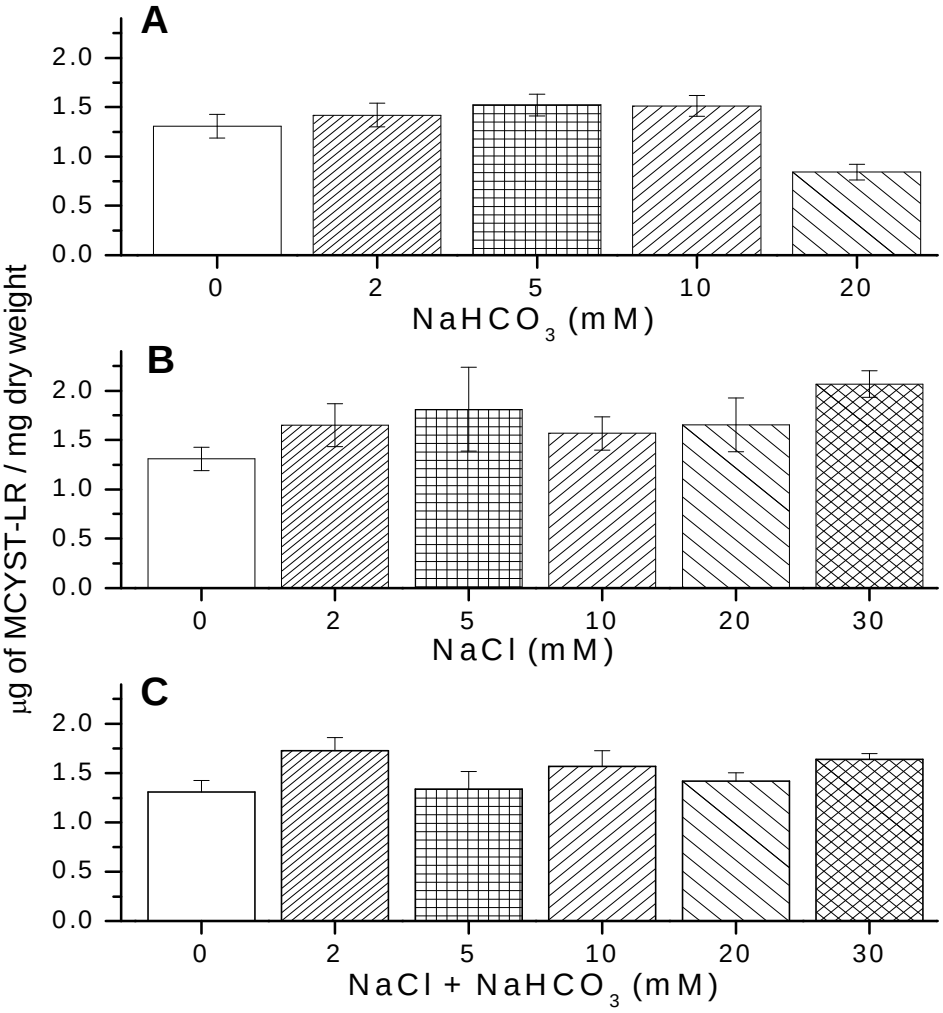

Figure 2.4
The effect on cultures of *M.aeruginosa* PCC7820 MCYST-LR levels when subjected to increasing concentrations (A) $NaHCO_3$, (B) NaCl and (C) a combination of NaCl +$NaHCO_3$ in equal portions over a period of 6 weeks.

The extracellular MCYST results (Table 2.12) were also very interesting, but did not follow the same pattern as seen in the previous experiment where as the concentration of $NaHCO_3$ increased the levels of extracellular MCYST increased. As in this experiment increasing the concentration did not affect the extracellular proportion and no extracellular MCYST was able to

be detected. Increasing levels of NaCl resulted in no extracellular MCYST being detected similar to the findings in the previous experiment, but much lower levels of extracellular MCYST were detected in the condition subjected to both NaCl and $NaHCO_3$ when compared to those detected in the previous experiment at 10 mM of the combined condition.

Condition	Intracellular		Extracellular		Total
	%	µg / ml	%	µg / ml	µg / ml
Control	100	1.80	N/D	N/D	1.80
$NaHCO_3$ 2 mM	100	1.89	N/D	N/D	1.89
$NaHCO_3$ 5 mM	100	2.06	N/D	N/D	2.06
$NaHCO_3$ 10 mM	100	1.96	N/D	N/D	1.96
$NaHCO_3$ 20 mM	100	0.69	N/D	N/D	0.69
$NaHCO_3$ 30mM	N/G	N/G	N/G	N/G	N/G
NaCl 2 mM	88	2.15	12	0.30	2.45
NaCl 5 mM	93	2.13	7	0.17	2.30
NaCl 10 mM	100	2.18	N/D	N/D	2.18
NaCl 20 mM	100	2.24	N/D	N/D	2.24
NaCl 30 mM	100	2.65	N/D	N/D	2.65
NaCl+$NaHCO_3$ 2 mM	84	2.22	16	0.42	2.64
NaCl+$NaHCO_3$ 5 mM	100	2.01	N/D	N/D	2.01
NaCl+$NaHCO_3$ 10 mM	83	1.95	17	0.39	2.34
NaCl+$NaHCO_3$ 20 mM	83	1.72	17	0.36	2.08
NaCl+$NaHCO_3$ 30 mM	100	1.93	N/D	N/D	1.93

Table 2.12
Effect of NaCl, $NaHCO_3$ and NaCl + $NaHCO_3$ on the levels of MCYST-LR both intracellularly and extracellularly shown as a percentage (n = 4, % mean in parenthesis, N/D = not detected, N/G = no growth).

2.3.6 Effect of NaCl, $NaHCO_3$ and combinations of these from 2 - 30mM on MCYST levels

In the previous experiment no extracellular MCYST was detected in the control and $NaHCO_3$ conditions, which is unusual as in all previous experiments MCYST were detected under these

conditions. It was also noted that in the combined tests that extracellular MCYST was detected in some and not in others as might be expected. Also in the highest concentration of $NaHCO_3$ no grow was observed and therefore it was decided to repeat the experiment to verify the results obtained in section 2.3.5.

The effect of increased Na^+ ions on MCYST levels in cultures of *M. aeruginosa* PCC7820 were tested by growing in media containing both NaCl and $NaHCO_3$ together; these were then compared to cultures grown in the presence of either NaCl or $NaHCO_3$. The effect of these conditions on the biomass produced during a 6 week growth period (Table 2.13) show that the presence of NaCl or $NaHCO_3$ separately causes an increase in biomass compared to the control cultures. A combination of both NaCl and $NaHCO_3$ together however has no increase on the biomass above levels observed in the controls; a reduction in biomass was only observed in the highest concentration of 30 mM NaCl and $NaHCO_3$.

Condition	Biomass		MCYST-LR	
	Mean mg / ml	± S.D	µg / mg	%*
Control	0.86	0.15	3.37	-
$NaHCO_3$ 2 mM	1.12	0.17	2.87	-14.9
$NaHCO_3$ 5 mM	1.06	0.08	2.97	-11.9
$NaHCO_3$ 10 mM	1.05	0.09	3.46	2.5
$NaHCO_3$ 20 mM	0.91	0.14	2.05	-39.3
$NaHCO_3$ 30 mM	0.73	0.08	1.16	-65.7
NaCl 2 mM	0.76	0.07	4.48	32.9
NaCl 5 mM	1.01	0.20	4.38	29.9
NaCl 10 mM	0.97	0.15	3.75	11.2
NaCl 20 mM	1.03	0.05	3.98	17.9
NaCl 30 mM	1.08	0.11	3.70	9.7
NaCl + $NaHCO_3$ 2 mM	0.87	0.11	4.37	29.5
NaCl + $NaHCO_3$ 5 mM	0.89	0.08	4.06	20.4
NaCl + $NaHCO_3$ 10 mM	0.95	0.05	4.13	22.4
NaCl + $NaHCO_3$ 20 mM	0.77	0.23	3.02	-10.6
NaCl + $NaHCO_3$ 30 mM	0.53	0.07	1.47	-56.6

Table 2.13
Biomass weights for cultures of *M.aeruginosa* PCC7820 cultures tested over a variety of concentrations subjected to both; NaCl + $NaHCO_3$, $NaHCO_3$ and NaCl at concentrations ranging from 2 mM to 30mM. (±S.D = standard deviation, n = 4) *Percent variation when compared to control.

The results for the intracellular analysis (Figure 2.5) show that in the presence of increased inorganic carbon through the addition of $NaHCO_3$ a decrease in the MCYST levels is clearly shown in the higher concentrations. This was not observed in

the presence of NaCl where increasing the concentration showed no effect on the levels on MCYST present after 6 weeks of growth. Increasing levels of NaCl and $NaHCO_3$ together at low concentrations up to 10 mM caused and increase in MCYST levels. At concentrations higher than 10 mM a gradual decrease was observed similar to the trend observed in the presence of $NaHCO_3$ alone suggesting this is as a direct result of increased inorganic carbon.

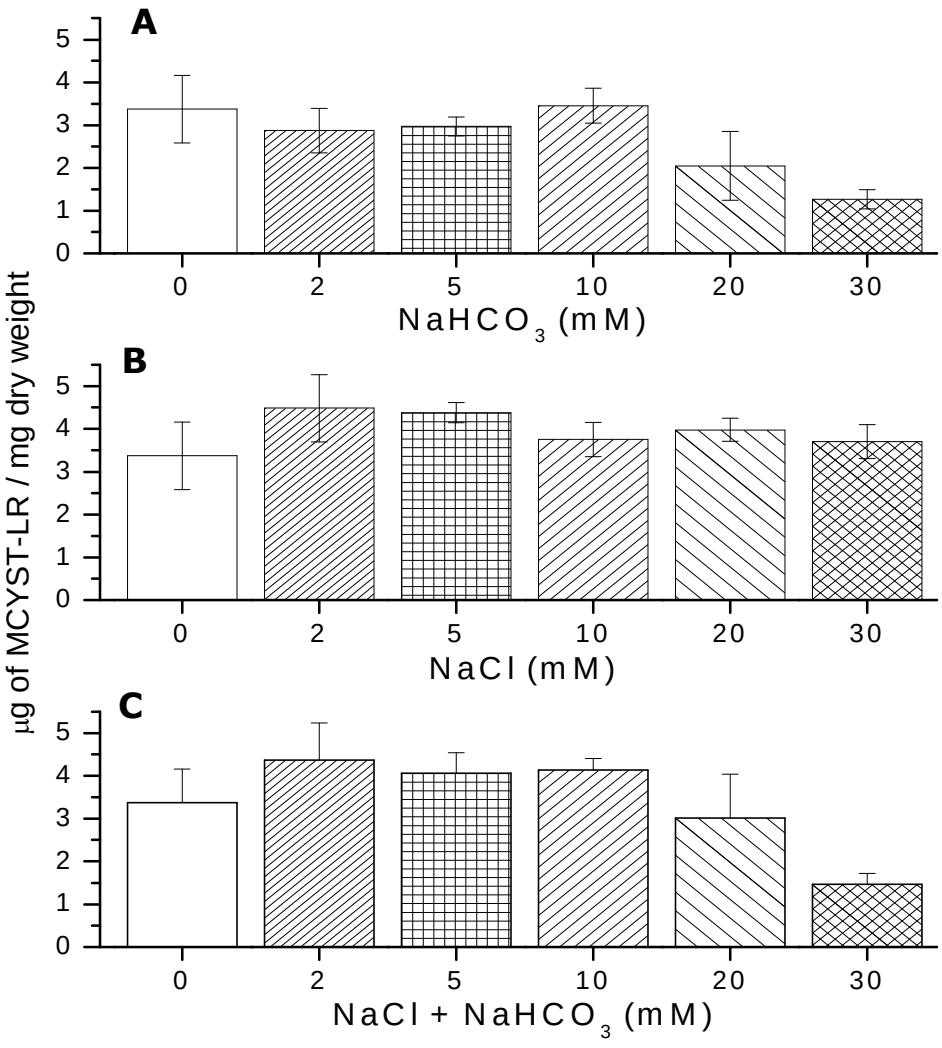

Figure 2.5
The effect on cultures of *M.aeruginosa* PCC7820 MCYST-LR levels when subjected to increasing concentrations (A) $NaHCO_3$, (B) NaCl and (C) a combination of both NaCl and $NaHCO_3$ in equal portions over a period of 6 weeks.

The effect of these three conditions on the extracellular MCYST (Table 2.14) also proved interesting as in the presence of increasing $NaHCO_3$ either alone or combined with NaCl + $NaHCO_3$ together the lower concentrations of 2, 5 and 10 mM all had their intra and extracellular percentages similar to those found in the controls with ~60% intracellularly and ~40% extracellularly. Then as the concentration increases to 20 mM the intracellular levels drop to ~45% and the extracellular proportion increases to ~55%. This continues in the 30 mM condition with the proportion of intracellular decreasing to ~30% and the extracellular proportion increases to ~70%. This increase in extracellular MCYST can only be as a result of the increase in inorganic carbon as this was not observed in the NaCl cultures but was observed in the $NaHCO_3$ cultures. Such increases in extracellular MCYST are probably due to cell lysis as (Orr *et al.*, 2004) found no significant increase in membrane permability to MCYST as a result of increased salt.

Condition	Intracellular		Extracellular		Total
	%	µg / ml	%	µg / ml	µg / ml
Control	64	2.82	36	1.56	4.38
$NaHCO_3$ 2 mM	67	3.24	33	1.60	4.84
$NaHCO_3$ 5 mM	62	3.14	38	1.92	5.06
$NaHCO_3$ 10 mM	70	3.60	30	1.52	5.14
$NaHCO_3$ 20 mM	45	1.94	55	2.40	4.33
$NaHCO_3$ 30mM	26	0.91	74	2.65	3.56
NaCl 2 mM	72	3.35	28	1.30	4.65
NaCl 5 mM	72	4.42	28	1.68	6.10
NaCl 10 mM	68	3.61	32	1.70	5.31
NaCl 20 mM	71	4.08	29	1.64	5.71
NaCl 30 mM	66	4.02	34	2.05	6.08
NaCl + $NaHCO_3$ 2 mM	68	3.71	32	1.77	5.48
NaCl + $NaHCO_3$ 5 mM	67	3.59	33	1.80	5.39
NaCl + $NaHCO_3$ 10 mM	77	3.90	23	1.13	5.03
NaCl + $NaHCO_3$ 20 mM	45	2.17	55	2.65	4.83
NaCl + $NaHCO_3$ 30 mM	30	0.79	70	1.84	2.63

Table 2.14
Effect of NaCl, $NaHCO_3$ and NaCl + $NaHCO_3$ on the levels of MCYST-LR both intracellularly and extracellularly shown as a percentage (n = 4, % mean in parenthesis).

2.3.7 Effect of buffered media on MCYST levels in the presence of increased inorganic carbon

Research by (Jähnichen *et al.*, 2001) and others have unequivocally shown that MCYST synthesis is highest at the end of the exponential phase suggesting that the history of the inoculum may be critical to the subsequent experiment.

Throughout previous results there is often a difference in findings for the same conditions between different experiments. In these studies the experimental variables had been carefully standardized with the exception of inoculation age and exact cell density. To improve the consistency a growth curve (Section 2.2.13) for *M. aeruginosa* PCC7820 cells was carried out, to determine when cells would reach exponential phase the results (Figure 2.6) showed that under these growth conditions cultures of M.aeruginosa PCC7820 reach log phase at nine days from sub culturing. Therefore all experiments were prepared from cultures in their ninth day of growth after inoculation.

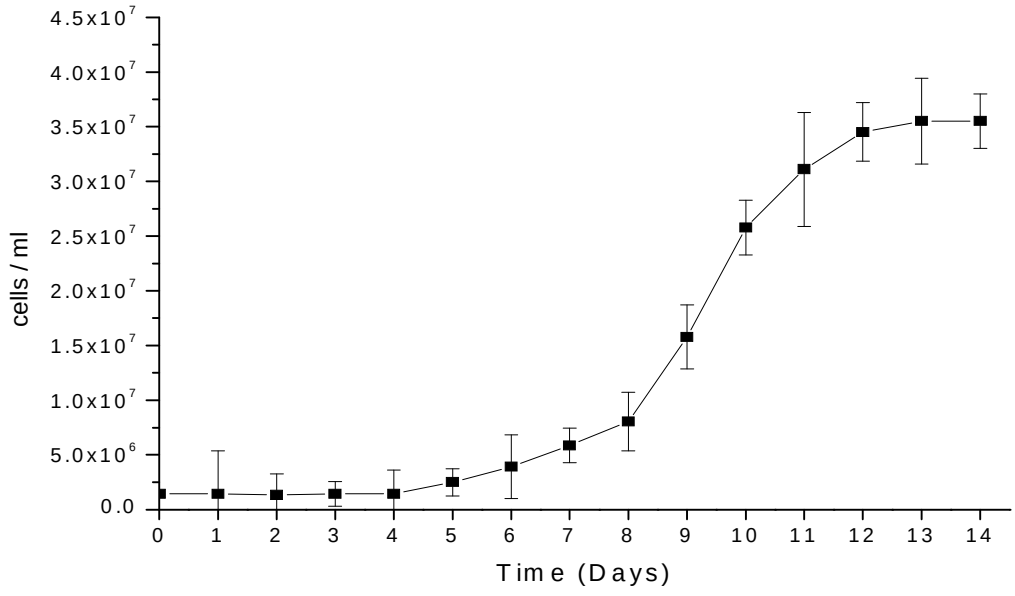

Figure 2.6
Growth curve of *M. aeruginosa* PCC7820 over a period of 14 days from a starting cell density of 1.4 x 10^6 cells / ml.

Furthermore the inoculated cells would also be at the same stage in the cell cycle and measuring cell density (section 2.2.17) standardized the starting inoculum to ~ 1.5 million cells / ml. Additional parameters were also assessed throughout this investigation, since research by (Kotak *et al.*, 1995) identified a positive correlation between MCYST, biomass, chlorophyll a, pH and others parameters. Therefore to assist in our understanding of how and what is being affected in relation to any changes in MCYST content cell counts, chlorophyll a, protein and pH were analysed to correlate MCYST changes with these parameters.

The addition of $NaHCO_3$ to the media causes an increase in the pH of the media and could potentially influence the proportions of intra and extracellular MCYST, therefore a buffer was introduced into the media in an attempt to maintain the pH at 7.60 throughout the experiment. The presence of HEPES buffer increased the biomass (Table 2.15) in both the HEPES control and HEPES with $NaHCO_3$ (20 mM) to over double the biomass observed in the control with no HEPES buffer present. The presence of HEPES buffer was found to increase the level of biomass and caused a significant reduction in intracellular MCYST. However when the extracellular MCYST was analysed and taken in to account it is quite clear that this reduction is either a result of cellular export or MCYST leakage (Table 2.16).

Condition	Biomass		Intracellular MCYST-LR	
	Mean mg / ml	±S.D	µg / mg	%*
Control	0.70	0.21	3.33	-
HEPES Control	1.71	0.11	0.45	-86.4
HEPES $NaHCO_3$ 20 mM	1.92	0.19	1.10	-67.1

Table 2.15
Biomass weights for cultures of *M.aeruginosa* PCC 7820 cultures tested in media buffered with HEPES buffer and tested with $NaHCO_3$ 20 mM. (±S.D = standard deviation, n = 4) *Percent variation when compared to control.

In addition to the standard HPLC analysis of the intra and extracellular MCYST levels, the chlorophyll a, protein and cell counts were carried out to determine how these relate to the levels of MCYST present after 6 weeks of growth. The intracellular MCYST levels have also been calculated in relation to these factors (Figures 2.7 to 2.10), also the three other MCYSTs produced have been reported to give a complete understanding how each of the MCYST levels have been affected. In figure 2.7 the intracellular MCYST levels are considerably affected by the presence of HEPES buffer with approximately a 7 fold decrease in MCYST-LR observed. Similar reductions were also observed in the other three MCYST produced, but the addition of $NaHCO_3$ causes an increase in three out of the four MCYSTs produced with only MCYST-LY not being increased by the presence of increased inorganic carbon when reported in relation to the dry weight of cells. The result

have also been calculated as µg / cell, µg / mg chlorophyll a and µg / mg protein, with all showing a similar trend in three of the four MCYSTs. Only MCYST-LY behaved differently and was found to decrease in the presence of increased inorganic carbon in relation to the control and the HEPES control. MCYST-LR, LW and LF were all observed to have increased in the presence of increased inorganic carbon when HEPES was present, but the levels detected in most cases were not any higher than the controls with no buffer present which may suggest that increased inorganic carbon only cancels out the effect of the HEPES buffer and does not increase the level of MCYST.

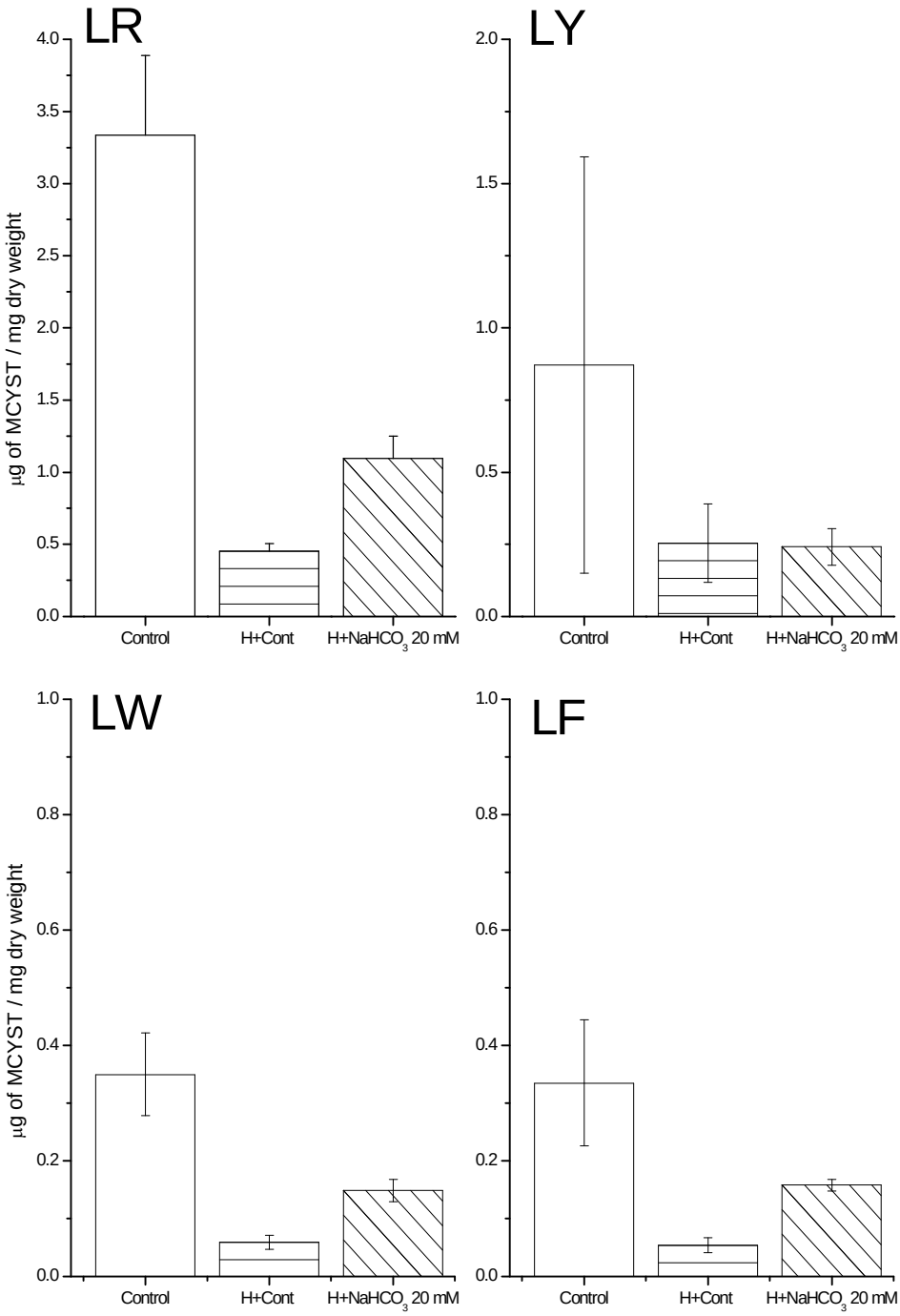

Figure 2.7
Comparison of how the presence of 25 mM HEPES buffer (H+Cont) and 25 mM HEPES with 20 mM $NaHCO_3$ (H+$NaHCO_3$ 20 mM) effects the levels of MCYST produced by *M.aeruginosa* PCC7820 over a period of 6 weeks in relation to mg dry weight.

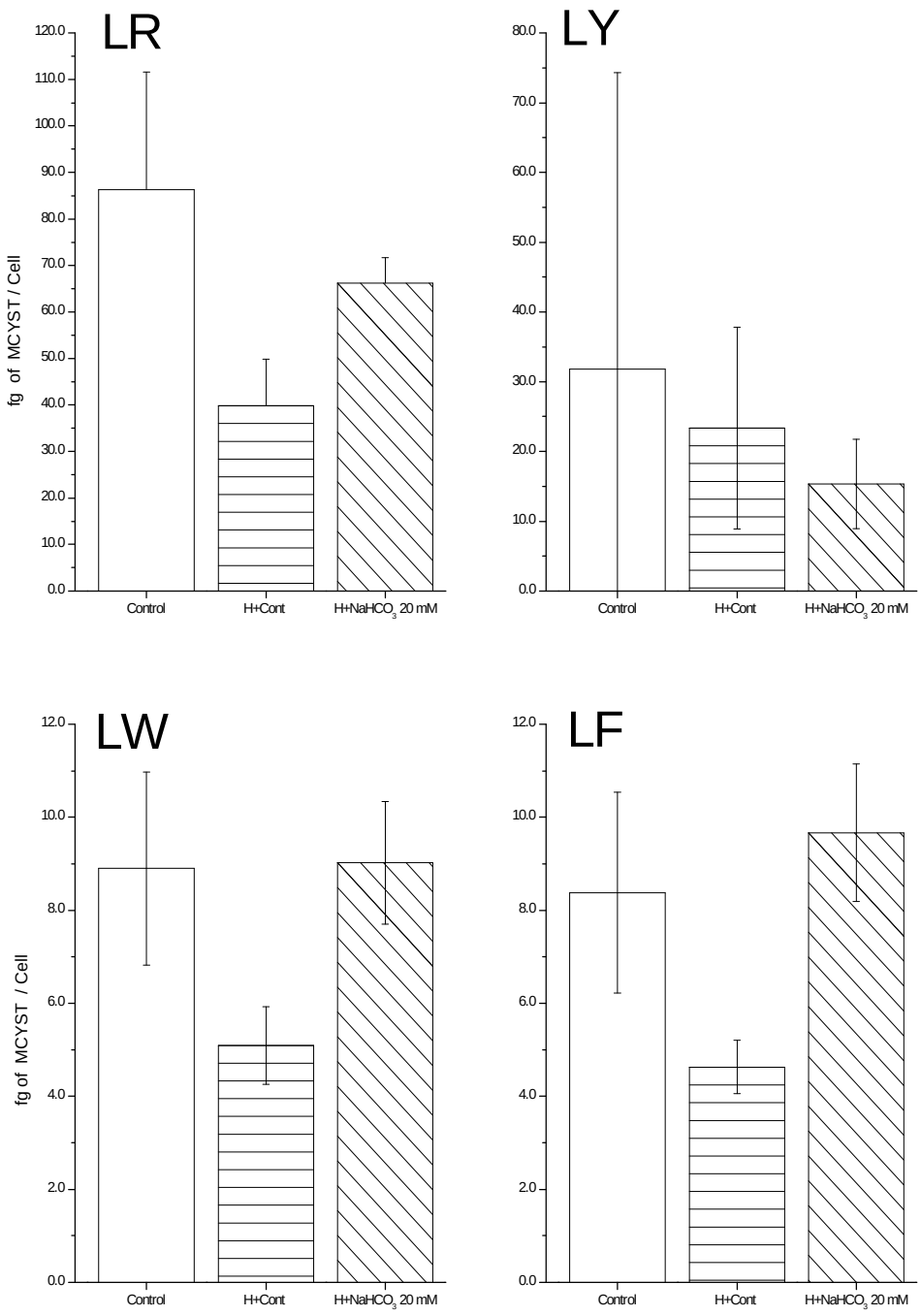

Figure 2.8
Comparison of how the presence of H+Cont and H+$NaHCO_3$ 20 mM effects the levels of MCYST produced by *M.aeruginosa* PCC7820 over a period of 6 weeks in relation to cells.

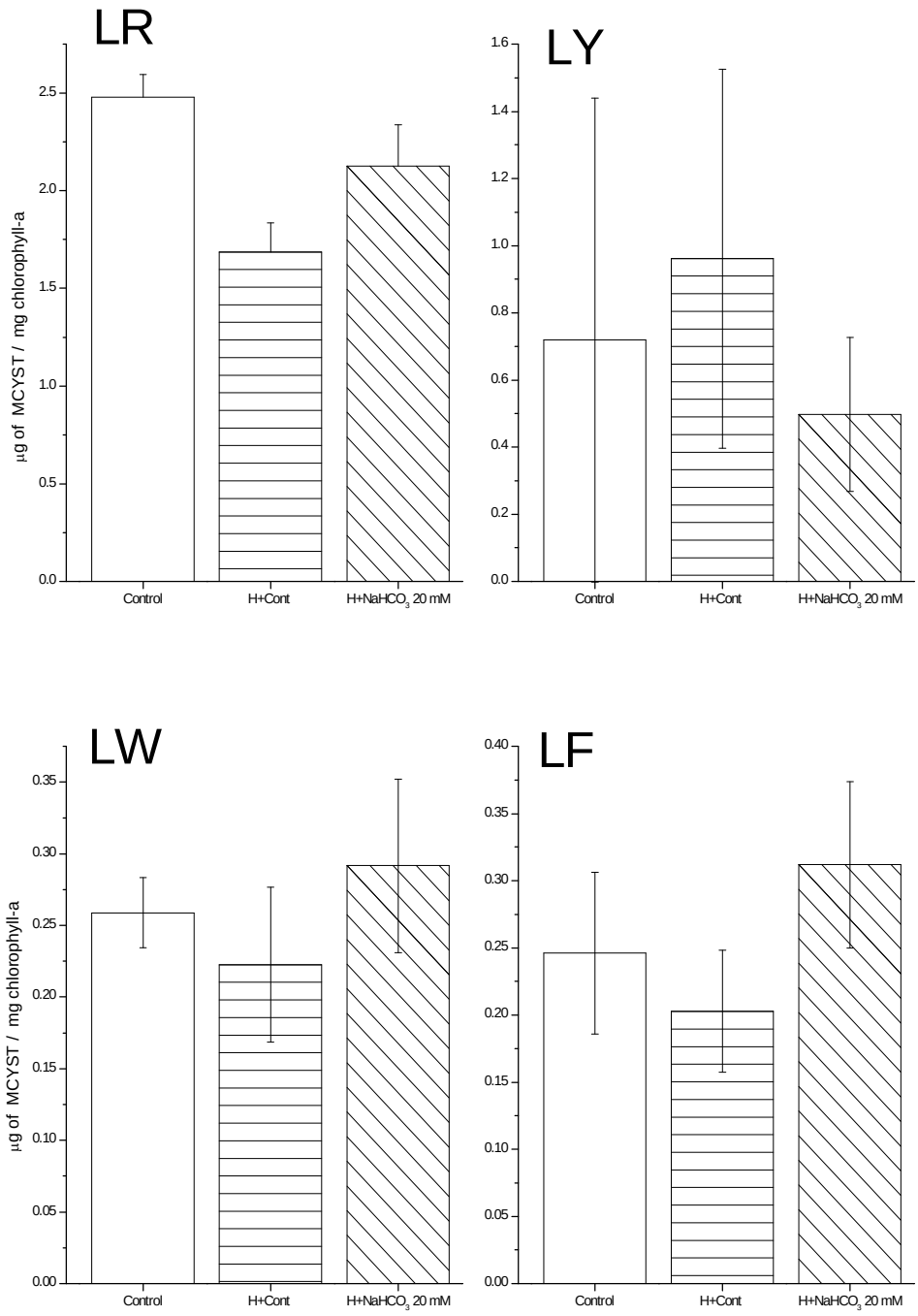

Figure 2.9
Comparison of how the presence of H+Cont and H+$NaHCO_3$ 20 mM effects the levels of MCYST produced by *M.aeruginosa* PCC7820 over a period of 6 weeks in relation to mg chlorophyll-a.

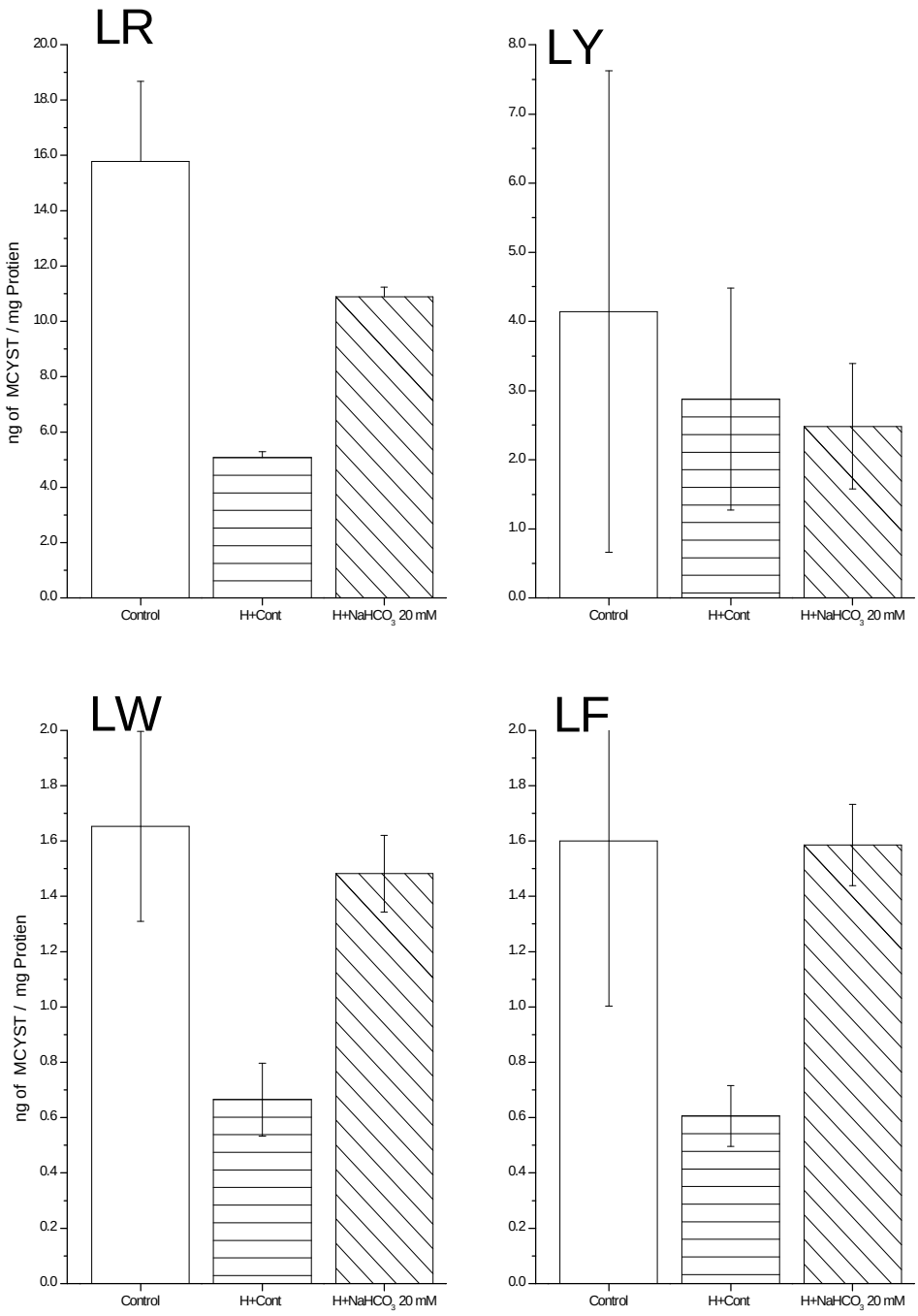

Figure 2.10
Comparison of how the presence of H+Cont and H+$NaHCO_3$ 20 mM effects the levels of MCYST produced by *M.aeruginosa* PCC7820 over a period of 6 weeks in relation to mg protein.

In this experiment the presence of 25 mM HEPES buffer was found to be unable to maintain a pH at ~7.60 especially under increased inorganic carbon conditions, as the pH of the media when HEPES + $NaHCO_3$ (20 mM) were present actually increased compared to that of the control. Although HEPES was able to maintain the pH in the HEPES control cultures (Figure 2.11), the effect on cell number and chlorophyll a content was considerably reduced when compared to those of the control and HEPES + $NaHCO_3$ (20 mM). However the presence of HEPES was not found to cause a similar reduction in the protein levels. It can therefore be assumed that although cell numbers have decreased the amount of protein per cell has actually increased as a result of HEPES being present.

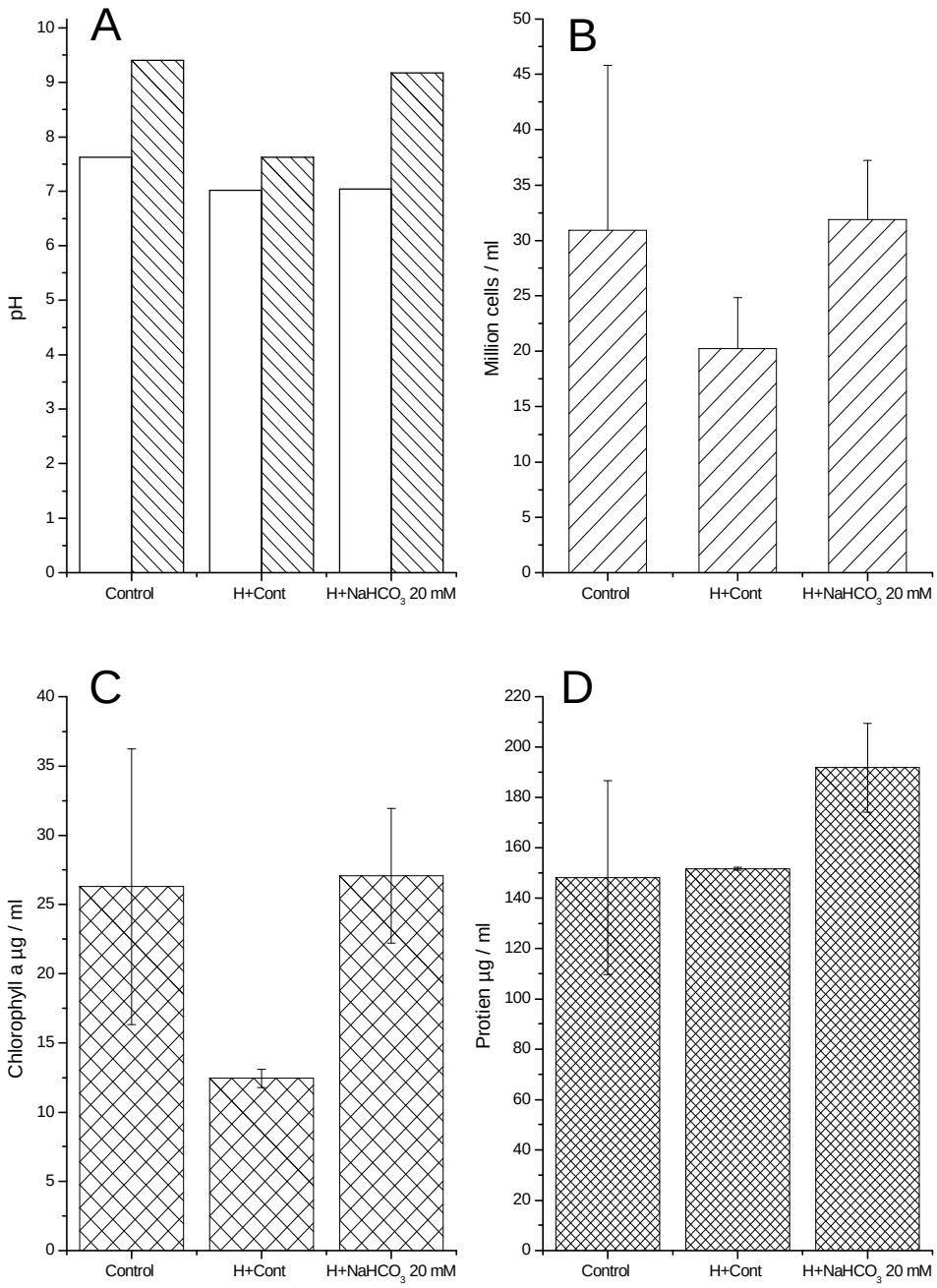

Figure 2.11
(A) Comparison between inoculation pH and after 6 weeks growth in controls, H+Cont and H+$NaHCO_3$ 20 mM at the start ☐ and end of the experiment ▨. The presence of H+Cont and H+$NaHCO_3$ 20mM were compared to the control containing only BG-II for (B) cell numbers, (C) Chlorophyll a and (D) Protein content.

MCYST (Table 2.16) by buffered media were found to be quite significant as in the presence of HEPES 74% of MCYST was detected extracellularly in the HEPES control. Such high levels of extracellular MCYST were however not detected when increased $NaHCO_3$ and HEPES were together and the proportions of MCYST intracellularly or extracellularly were more aligned with those detected in the control with no HEPES present. Therefore it can only be assumed that the presence of increased inorganic carbon in the form of $NaHCO_3$ reduces cellular stress or export of MCYST from within the cell, resulting in levels more in line with cultures grown in non-buffered media.

Condition	**Intracellular**		**Extracellular**		**Total**
	%	µg / ml	%	µg / ml	µg / ml
Control	71.0	2.42	29.0	0.99	3.41
H + Control	25.8	0.77	74.2	2.21	2.98
H + $NaHCO_3$ 20 mM	63.4	2.09	36.6	1.21	3.30

Table 2.16
The effect of HEPES buffer on the mean percent and µg / ml of culture for MCYST intra- and extra-cellular levels either alone or in the presence of 20 mM $NaHCO_3$ compared to a control culture of *M. aeruginosa* PCC7820.

2.4 Conclusion

The effects on MCYST observed by (Graham *et al.*, 2001) through the addition of $NaHCO_3$ to increase the availability of inorganic carbon, showed significant reductions in MCYST levels. However, that research never investigated if these findings were as a direct result of the increased inorganic carbon or as a result of increases in Na^+ ions. The data in the present study however shows that the presence of increased Na^+ ions had no effect on the levels of MCYST detected when concentration of Na^+ ions present are similar to those of 2 - 40 mM $NaHCO_3$, they are also not high enough to cause significant cell stress or lysis. One important finding however was that concentrations >40 mM of $NaHCO_3$ caused cell lysis while concentrations of NaCl up to 70 mM were tolerated in laboratory culture of *M.aeruginosa* PCC7820. It was also found that the use of HEPES buffer at 20 mM to maintain a pH of ~7.6 was unsuccessful when $NaHCO_3$ was present; and had a significant effect on the intracellular level of MCYST under standard inorganic carbon levels as three quarters of the total MCYST detected was extracellular.

The types of analysis carried out is also of major concern as more parameters provide a more detailed and in depth understanding of cell behaviour and factors affected during experiments. To date there is also no standard or definite way to

report MCYST levels as many of the early studies reported the level in relation to biomass and only more recently have some studies reported their findings as cell quota; the parameters investigated in this chapter have expanded to include cell number, chlorophyll a and protein together with a standardized inoculum. This data also clearly shows that the presence of increased available inorganic carbon can alter levels of MCYST, although further investigations with either air enriched with CO_2 or other methods for increasing available inorganic carbon may improve our understanding of MCYST function within the cell.

Chapter 3

Influence of CO_2 on MCYST levels

3.1 Introduction

Many studies have been performed to determine the factors that influence the toxicity of cyanobacteria, especially the elevation or suppression of MCYST levels. While environmental variables such as light, temperature and nutrients have been studied by a number of research groups, however studies have not investigated the influence of inorganic carbon on MCYST levels. Modifying inorganic carbon via addition of sodium bicarbonate (Chapter 2) indicated that MCYST levels were reduced although conclusive results were difficult to obtain. This was in part due to the alteration in the pH which could not be regulated by the use of buffers without altering the experimental findings. Also, the addition of inorganic carbon in the form of sodium bicarbonate altered Na^+ ion levels as well. It was therefore decided that the elevation of inorganic carbon using CO_2 was an appropriate alternative strategy.

The addition of CO_2 via sparging of gas into a liquid media is unlike the behaviour of many other gasses as it reacts to form a species as follows

$$\underset{}{CO_{2\,(AIR)}} \underset{\text{Rapid}}{\leftrightarrows} \underset{(\text{Dissolved})}{CO_2} + \underset{}{H_2O} \underset{\text{Slow}}{\leftrightarrows} \underset{\text{Carbonic acid}}{H_2CO_3} \underset{\text{Fast}}{\leftrightarrows} \underset{}{H^+ + HCO_3^-} \underset{\text{Fast}}{\leftrightarrows} \underset{\text{bicarbonate}}{2H^+ + CO_3^{2-}}$$

Active photosynthesis by cyanobacteria alters the equilibrium towards bicarbonate which in turn pushes the pH up. It is commonly observed in cyanobacterial cultures that pH rises to a pH of 9 - 11, and this also occurs in the natural environment. Conversely addition of CO_2 enriched air will result in a drop in pH.

Research was carried out to develop a suitably robust culture system for the inorganic carbon enrichment of MCYST producing cultures using CO_2 enriched air. The effect of elevated CO_2 on growth characteristics and MCYST levels were followed. This work may be of particular relevance since global atmospheric carbon levels have been observed to almost double (IPCC, 2001) in the last 100 years or so. If this trend continues it is imperative that we understand the impact these changes will have on the risk associated with toxic blooms. Most commonly the current focus is on the influence of elevated temperature as this is predicted to increase frequency and magnitude of blooms.

3.2 Methods

3.2.1 Effect of an elevated CO_2 atmosphere on growth and MCYST production in cultures of *M. aeruginosa*.

Conical flasks (500 ml) were prepared with 250 ml of sterile BG-II medium and inoculated at 1.5 x 10^6 cells / ml (Section 2.2.13 and 2.2.17) (cells were taken from a 9 day culture) with *M.aeruginosa* PCC 7820. They were incubated at 25 $^{\circ}$C in water a bath under continuous illumination (30 µmol s^{-1} m^{-2}). Flasks were exposed to an elevated CO_2 environment by placing the experimental set up in a clear polythene bag that was continually flushed with 5% CO_2 in air at a rate of 20 ml / min to create a positive pressure environment. Control cultures were prepared in the same way except, flushed continuously with air.

Cultures were sampled weekly (Ref. Chapter 2; section 2.2 where relevant) for biomass, cell numbers, chlorophyll a, intra and extracellular MCYST levels, protein and pH. Inorganic carbon analysis was performed at Aberdeen University Plant and soil science department, using a LabTOC (Pollution and Process monitoring, Seven Oaks, Kent, UK). Sample analysis was carried out using the UV digestion method developed by Pollution and Process monitoring, Uk and inorganic carbon levels calculated by subtracting the total organic carbon from the total carbon to give the total inorganic carbon. Analysis was carried out as per

the relevant methods in Chapter 2 section 2.2 with duplicate measurements for inorganic carbon in the four replicates of each experimental condition.

3.2.2 Effect of direct sparging with elevated CO_2 on growth (Version 1)

Flasks (500 ml) were prepared with 250 ml of BG-II medium (pH 7.6) to which tubing (3 ml I.D.) fitted with a stainless steel air stone was inserted. The flasks were autoclaved prior to being inoculated to give a starting concentration of log phase cells (1.5 x 10^6 / ml) (Section 2.2.13 and 2.2.17) then placed in a water bath at 25 ºC under constant illumination by fluorescent tubes delivering 30 µmol s^{-1} m^{-2} light. Flasks were sparged with 5% CO_2 in air at a rate of 20 ml / min which was split between the 4 replicate flasks. Control flasks were sparged with air at the same rate of 20 ml / min which was also split between the 4 replicate flasks (Figure 3.1). Cultures were sampled ever 3 days for biomass, cell number, chlorophyll a, intra and extracellular MCYST levels and protein analysis was carried out as per the relevant methods in Chapter 2 section 2.2.

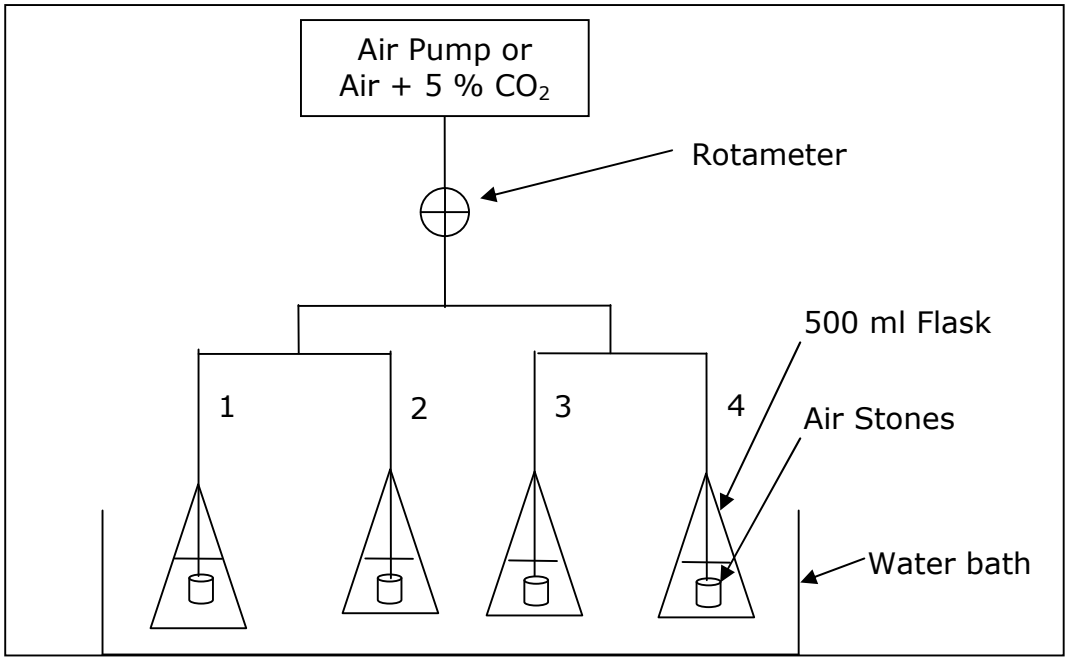

Figure 3.1
Version 1.0 Initial sparging experiments were set up as per the diagram above with either air or 5% CO_2 enriched air a rotameter was used to control the flow rate to 20 ml / min which was split between all 4 replicates.

3.2.3 Effect of direct sparging with elevated CO_2 on growth when air stones are removed (Version 2)

The experimental setup in section 3.2.2 was altered to achieve larger bubbles at the same flow rate, reducing the shear forces previously experienced when the bubbles reach the surface which possibly resulted in cell damage (Cherry *et al.*, 1992;Mirón *et al.*, 2003). All the parameters detailed in section 3.2.2 remained unchanged, except air stones were replaced with fine pipette tips (c200 µl size).

3.2.4 Effect of continuous sparging with 5% CO_2 in air on medium pH over a period of 24 hr

Using the experimental design (section 3.2.3), standard 500 ml flasks with the 250 ml of BG-II at pH 7.60 were connected to the system with no culture added. The flasks were then sparged continuously for a period of 24 hr and the pH recorded at regular intervals.

3.2.5 Effect of direct sparging with elevated CO_2 on growth using manifold gas regulation (Version 3)

Although gas flow was controlled by a rotameter, splitting the gas flow by dividing the tubing into equal lengths was unable to provide constant flow rates to each flask. Therefore after the rotameter a manifold was introduced to split the gas equally between cultures (note: all cultures had to have the same volume or gas flow resistance varied and altered flow rates). The connection between the flask and the gas regulating system was also altered as sample collection was difficult without compromising the sterility of the culture. Therefore as the silicon tubing (3.0x 1.5 mm I.D) within the flasks emerged through the cotton wool bung a medical cannula (Becton Dickinson Int. (BD), InsyteTM, Autoguard 1.1 x 48 mm) was inserted into the inside of the tubing prior to sterilization. This provided the opportunity to connect the gas regulating apparatus to the flasks with a

three port connecta (BD, 3 port Connecta) (Figure 3.2). This type of connection also simplified sample removal without compromising sterility and improved the connection with the sterile filter, the system schema is detailed below (Figure 3.3).

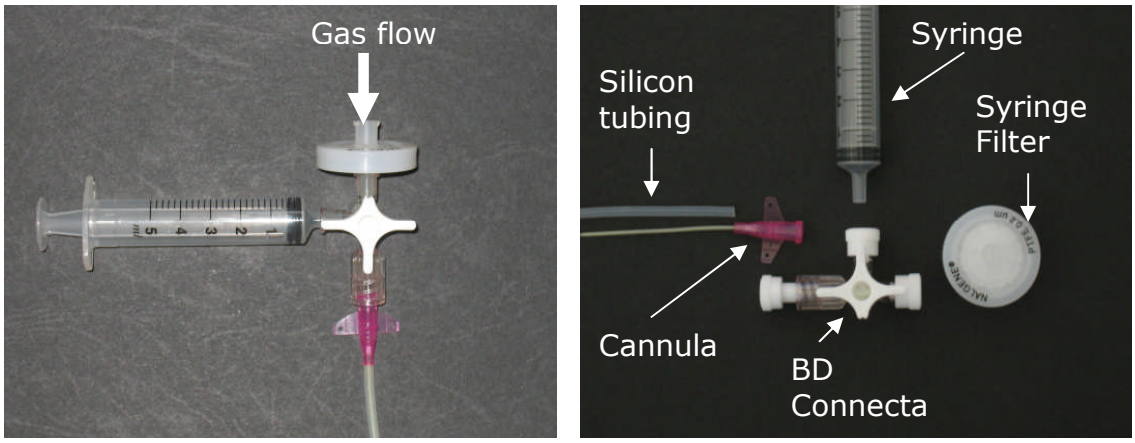

Figure 3.2
Detail of the components used to connect the gas flow system to the culture flask

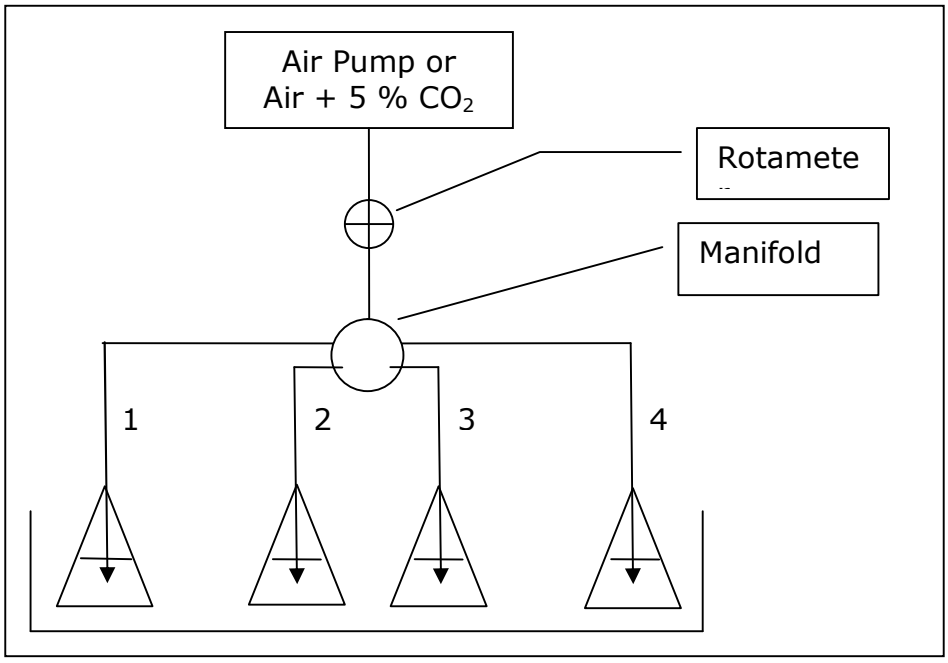

Figure 3.3
Version 3 set up where the gas was put through a rotameter and a manifold to control the flow rate and then split to supply each culture flask. Equal flow rate can be achieved if the length of tubing for 1-4 is the same and the equal volumes are present in each flask.

3.2.6 Effect of sparging with elevated CO_2 on growth using electronically controlled gas regulation (Version 4)

Although version 3 design improved the consistency of gas delivery in each culture flask, the manifold construction was not very robust. Therefore a new manifold was constructed, with hollow partially treaded brass rods used to replace pipette tips, making the manifold more stable and easier to move without the possibility of leaking. Also instead of the cultures being supplied with a constant supply of air enriched with 5 % CO_2 the sparging was reduced to 1 hr out of every 24 hr or for a 1 hr period every 12 hrs. Providing cultures with 1 hr of sparging every 12 hr over the course of the experiment (35 days) proved very labour intensive. Therefore further modifications were carried out to electronically control the system, switch gasses and shut down sparging if required. In order to do this a timer controlling three solenoids was introduced and a number of manifolds increasing the number of experimental conditions which can be tested at once (Figure 3.4). The system worked by running with solenoids A and C open to supply air to all the cultures, when the timer switched over for the desired sparging with air enriched with 5 % CO_2 solenoids A and C closed and solenoid B opened. The reverse happened when switching back to air only. An example of the experimental schema can be seen in Figure 3.5 where one

manifold (2) supplies 8 samples in one water bath with the controls in a second bath with a separate manifold (1) (Figure 3.4).

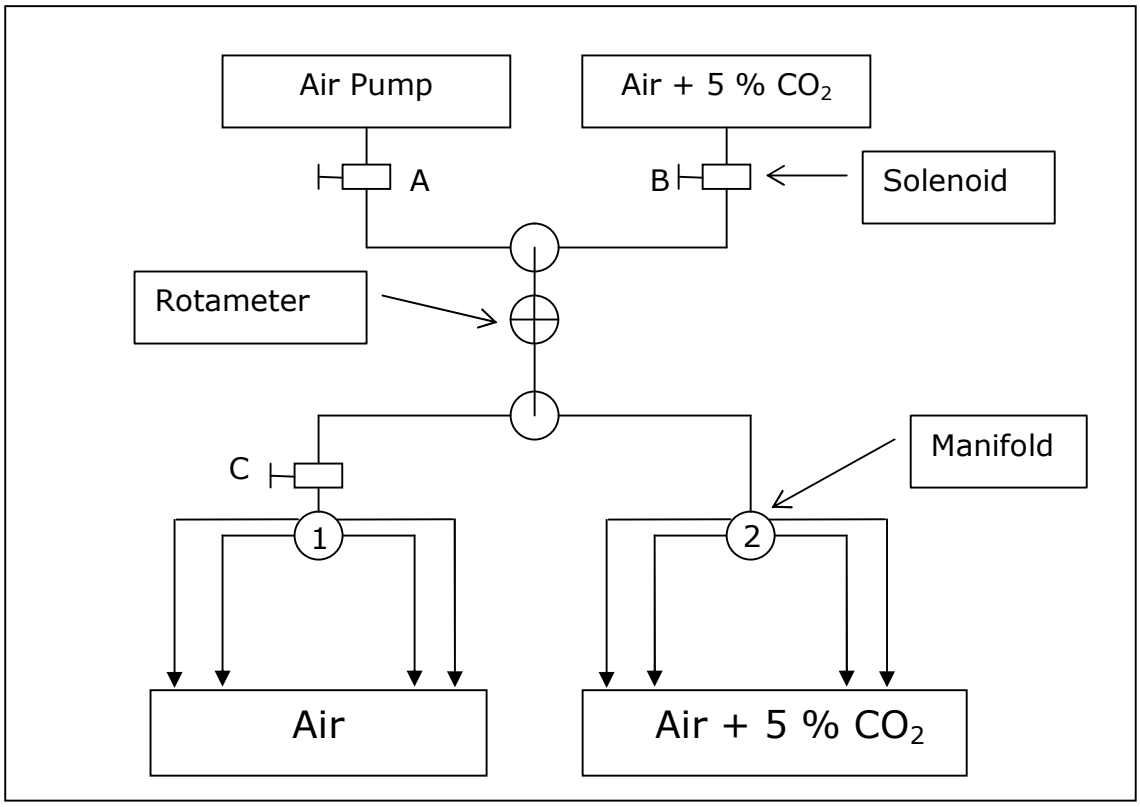

Figure 3.4
Version 4 an electronically controlled system controlled by a timer and solenoids (A – C) to switch from air to air enriched with 5 % CO_2 and close lines to the controls. In addition to this schema additional manifolds (1 – 2) can be added providing the possibility of testing more than one condition at a time provided all flow rates are consistent. All experiments were carried out using a gas flow rate of 20 ml / min.

Figure 3.5
Experimental set up using version 4 and 500 ml conical flasks with 250 ml of culture in each.

3.3 Results and discussion

3.3.1 Effect of an elevated CO_2 environment on the level of MCYST in *M. aeruginosa* strain PCC7820

As previous research into MCYST production has mainly focused on either nutrients or light availability (Sivonen, 1996) the effect of inorganic carbon has not been extensively studied. This experiment was designed to directly study the effect of enhancing the atmospheric conditions by the use of air enriched with 5 % CO_2. The experiment was set up as per section 3.2.1 in which cultures were placed in water baths and enclosed in clear plastic bags with different air conditions flowing through. Cultures incubated within the enclosed system grew poorly after the first few weeks and the experiment had to be terminated. However, the findings from this study did support the idea that elevated CO_2 increased levels of MCYSTs. This was observed for the main MCYST variants (LR, LW and LF) identified by method detailed in Chapter 2 Section 2.2.6 and although a similar finding was observed for MCYST-LY the data was highly variable. Although this experiment was terminated the results from the first three sample points are shown in Figure 3.6 and Figure 3.7. The artificial environment used in this experiment was found to have a considerable problem with condensation and variation between the replicates was observed. Therefore the

experimental design was altered so that each culture would be sparged directly and the system not enclosed by a clear polythene bag; eliminating condensation previously observed in the previous experiment. Direct sparging of each culture would also ensure an increase in CO_2 absorption within the media and increase its inorganic carbon level.

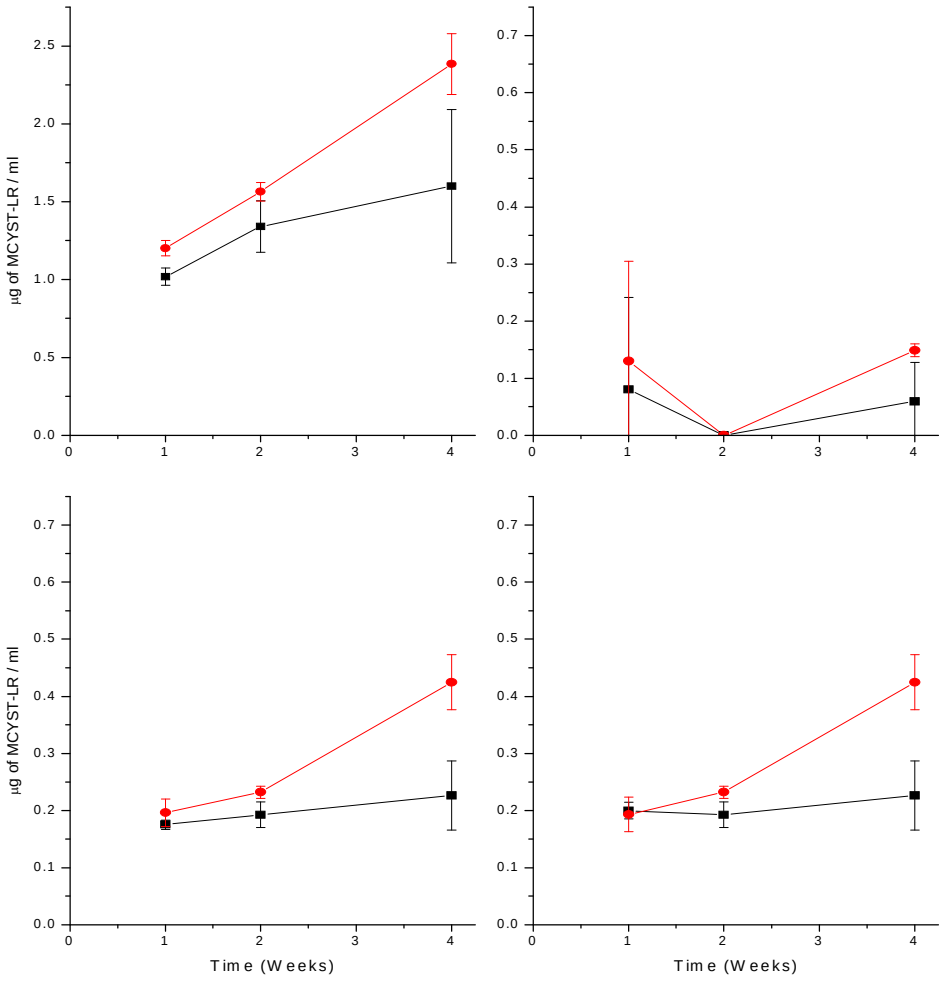

Figure 3.6
Effects of an enclosed atmosphere with increased CO_2 on MCYST levels calculated as µg / ml of culture (A) MCYST-LR, (B) MCYST-LY, (C) MCYST-LW and (D) MCYST-LF. Air ■, Air + 5% CO_2 ● the error bars indicate ± SEM (standard error of the mean), *n*=4.

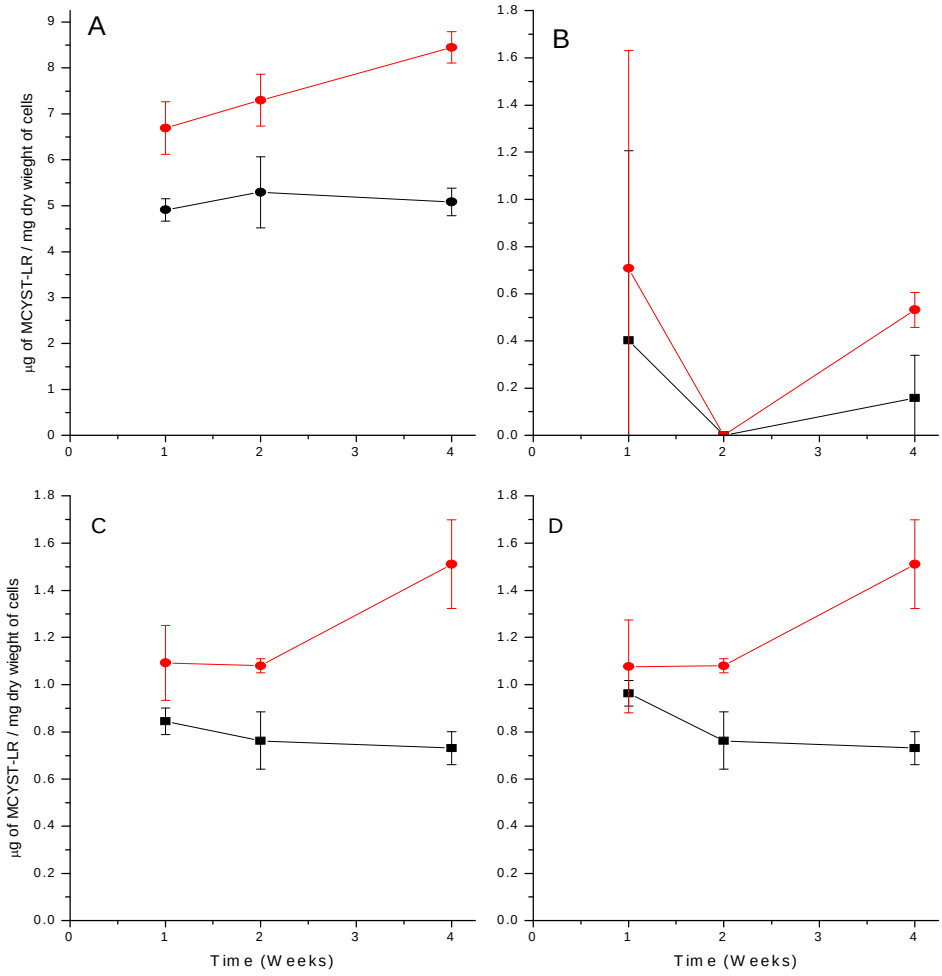

Figure 3.7
Effects of an enclosed atmosphere with increased CO_2 on MCYST levels calculated as µg / mg dry weight of cells (A) MCYST-LR, (B) MCYST-LY, (C) MCYST-LW and (D) MCYST-LF. Air ■, Air + 5% CO_2 ● the error bars indicate ± SEM (standard error of the mean), *n*=4.

3.3.2 Effect of direct sparging with air enriched with 5% CO_2 on MCYST levels using experimental design version 1

As a result of poor growth in the previous experiment where cultures of *M. aeruginosa* PCC7820 were grown in an enclosed atmosphere under positive pressure from either air enriched

with 5% CO_2 or air (Section 3.3.1). The experimental design was altered to a more direct approach in which each culture would be sparged directly; this new experimental set up is detailed in section 3.2.2. However after only one week the condition of the cultures started to decline and the experiment was terminated. The first weeks findings (results not shown) indicated that cultures sparged with air enriched with 5 % CO_2 had an increase in biomass, cell number, chlorophyll a and MCYST-LR when compared to those sparged with only air. This suggested that changes to the level of available inorganic carbon can directly affect the growth and the amount of MCYSTs in *M. aeruginosa* PCC7820. The rapid deterioration in the cultures after only one week was thought to be as a result of the experimental design. Previous research (Cherry *et al.*, 1992;Mirón *et al.*, 2003) proved that although sparging increased the transfer of gasses in to liquids many species are effected by the shear factors caused by bubbles bursting at the air-liquid interface. The rapid decline in growth observed could be as a result of similar shear factors as *M. aeruginosa* are well known for forming surface blooms and often congregate at the air-liquid interface. One other consideration was, the air stones used produced large numbers of very small bubbles which would result in a higher shear factor than fewer larger bubbles.

3.3.3 Effect of direct sparging with air enriched with 5% CO_2 on MCYST levels using experimental design version 2

As a result of our findings during the previous experiment in which shear factors were thought to be the cause of cell death, aeration stones were replaced with pipette tips to increase bubble size and limit shear stress (Section 3.2.3). Initially this change was thought successful, as cultures grew for a period of time but after the second time point cultures started to deteriorate and the experiment had to be terminated. In order to try and understand why prolonged growth was not being achieved, the pH of the media was tested under continuous sparging with 5% CO_2 in air as per section 3.2.4 and found the pH to drop to around 5.5 (Figure 3.8).

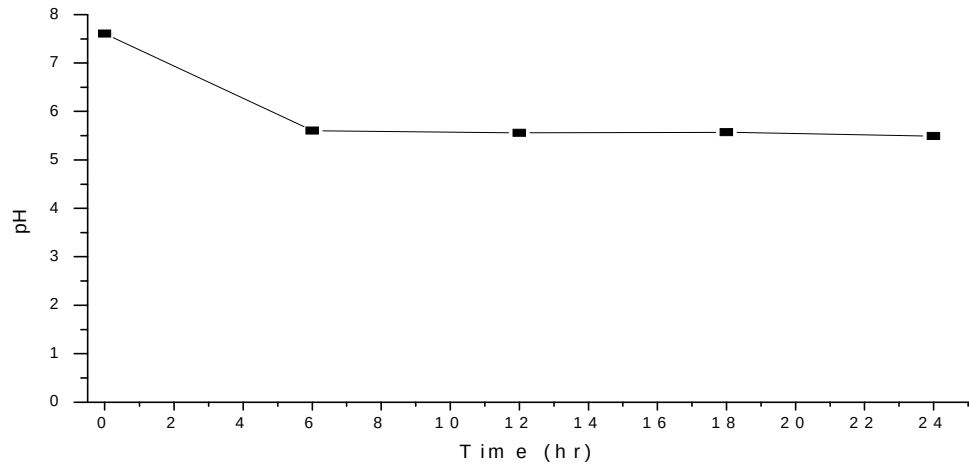

Figure 3.8
The effect of continuous sparging with 5% CO_2 enriched air at a flow rate of 20 ml / min on culture free BG-II media.

This low pH is unfavourable for growth in *M. aeruginosa* which usually inhabit an alkaline environment (Bañares-España *et al.*, 2006;Bañares-España *et al.*, 2007;Shapiro, 1984). It is therefore likely that continuous sparging with 5% CO_2 enriched air has resulted in cell death as a result of the stress from the low pH. It was also noted at this stage that some of the flow rates varied over time and could have resulted in some cultures receiving higher flow rates than others. As a result of these findings a manifold was introduced after the rotameter to eliminate fluctuations in flow rate between cultures, it was also found to be imperative that all the cultures had exactly the same volume, since the volume directly effects flow resistance.

3.3.4 Effect of sparging for 1 hr with 5 % CO_2 in air on growth and MCYST levels in cultures of *M.aeruginosa* PCC7820.

As a result of the previous findings, sparging with 5% CO_2 enriched air was reduced to only 1 hr in every 24 hr so that inorganic carbon could be increased without reducing the pH to ~5.5 (Figure 3.22), after this 1 hr the cultures were returned to continuous sparging with air as used in the controls. In Figure 3.9 the effect of exposure to 1 hr of increased inorganic carbon (CO_2) in every 24 hours showed no significant effect on *M. aeruginosa* biomass or cell count when compared to cultures only sparged with air.

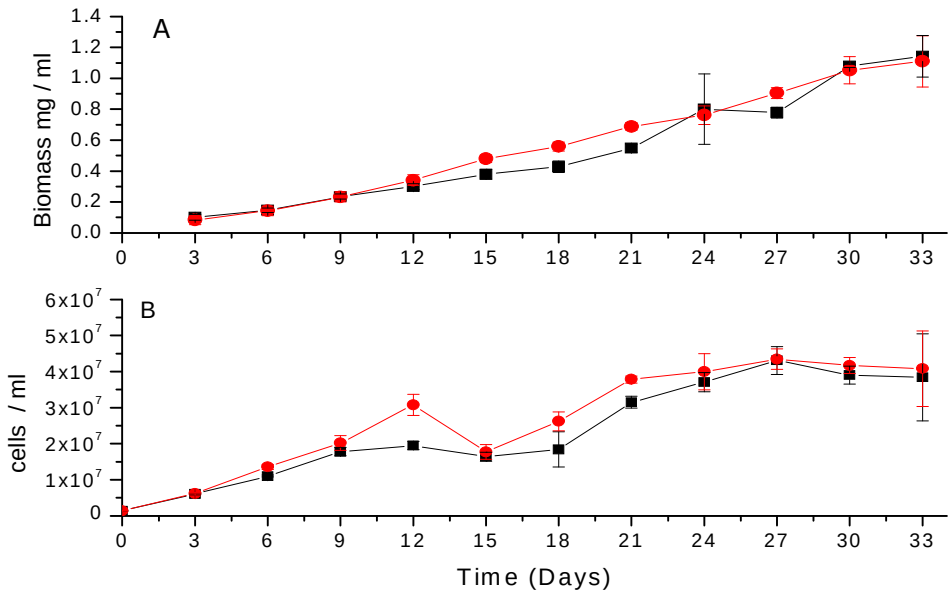

Figure 3.9
The effect on biomass (A) and cell counts (B) for cultures exposed to 1 hr with 5% CO_2 enriched air over a period of 33 days. Air ■ and 5% CO_2 enriched air ●.

The effects of increased inorganic carbon (CO_2) on MCYST levels are clearly shown in Figure 3.10 where significant increases in the level of MCYST-LR were observed in cultures subjected to 1hr of 5% CO_2 enriched air in every 24 hours compared to those sparged with air only. The presence of increased inorganic carbon however was found to slightly decrease the level of MCYST-LY (Figure 3.11) as time progressed and the difference between the air sparged cultures and those sparged for 1 hr in every 24 hours with air enriched with 5% CO_2 was negligible. As for MCYST-LW and –LF (Figures 3.12 and 3.13) they followed a similar trend to that observed for MCYST-LR with increased MCYST detected when compared to cultures sparged with only

air. The slight reduction in MCYST-LY could be as a result of a change in light intensity, as the inoculating cultures were grown at a light intensity of 15 μmol s^{-1} m^{-2} and the experiment light intensity was increased to 30 μmol s^{-1} m^{-2}. It should also be noted that the protein time points below 12 days are not shown as the levels were below the sensitivity of the method and the sample errors were too large to include. The sample errors observed when measuring protein in the initial time points could be a result of the low cell density at the start of the experiment and this could probably have been avoided if a larger sample was tested in the initial stages. In the method used (Lowry *et al.*, 1951) one of the disadvantages is colour can vary with different proteins and colour is not always proportional to concentration. Although the process was calibrated using bovine serum albumin and linear regression and concentrations extrapolated. These results clearly identified that increasing the level of inorganic carbon with 1hr air enriched with 5% CO_2 resulted in an increase in the intracellular levels of MCYST-LR, -LW and –LF, when calculated in relation to the parameters analysed. Only MCYST-LY showed a reduction under elevated inorganic carbon conditions suggesting that it has been down regulated or being exported into the surrounding media. However this can not be the case as later analysis of the extracellular sample detailed later in this chapter showed no

significant difference between the two sparging conditions. A similar increase in MCYST content was also observed by (Codd *et al.*, 1988) who found a greater toxicity in cultures supplied with CO_2.

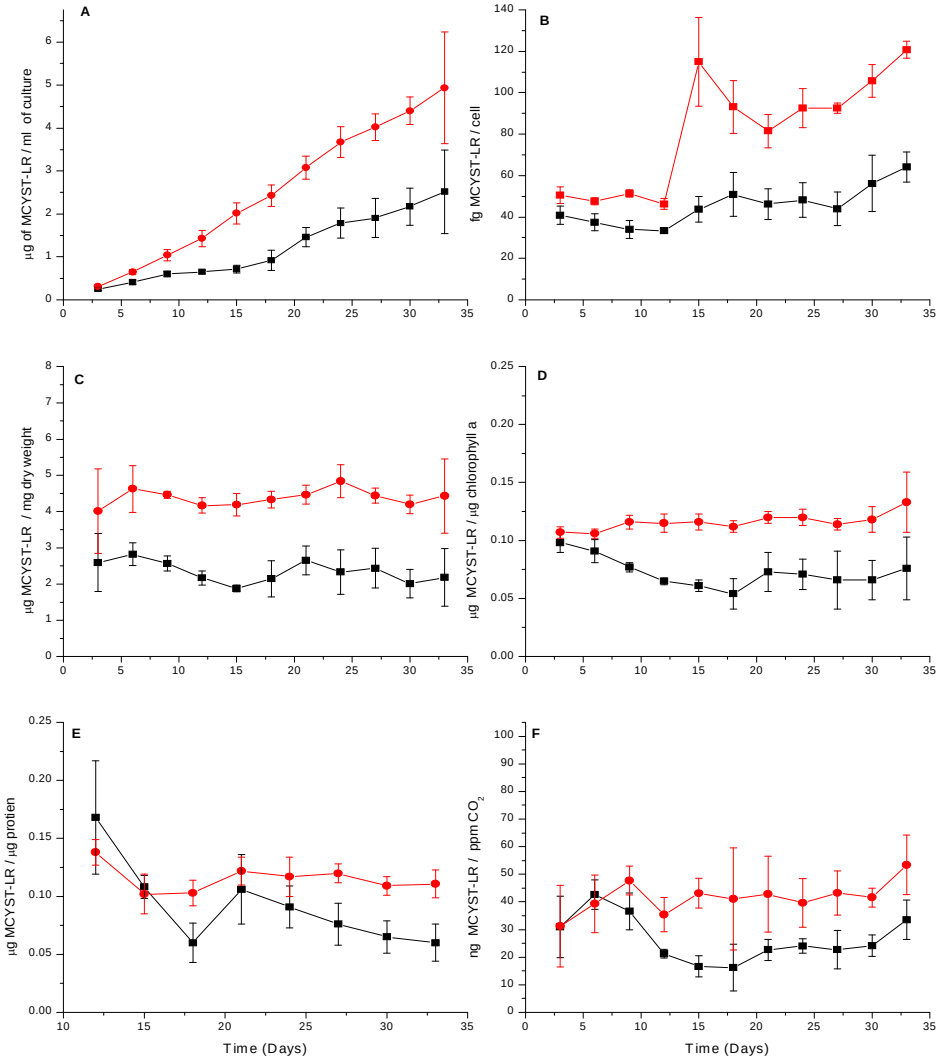

Figure 3.10
Effects of 1 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LR levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LR reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LR / ml of culture, (B) fg MCYST-LR / cell, (C) µg MCYST-LR / mg dry weight of cells, (D) µg MCYST-LR / µg chlorophyll a, (E) µg MCYST-LR / µg protein and (F) ng MCYST-LR / ppm CO_2.

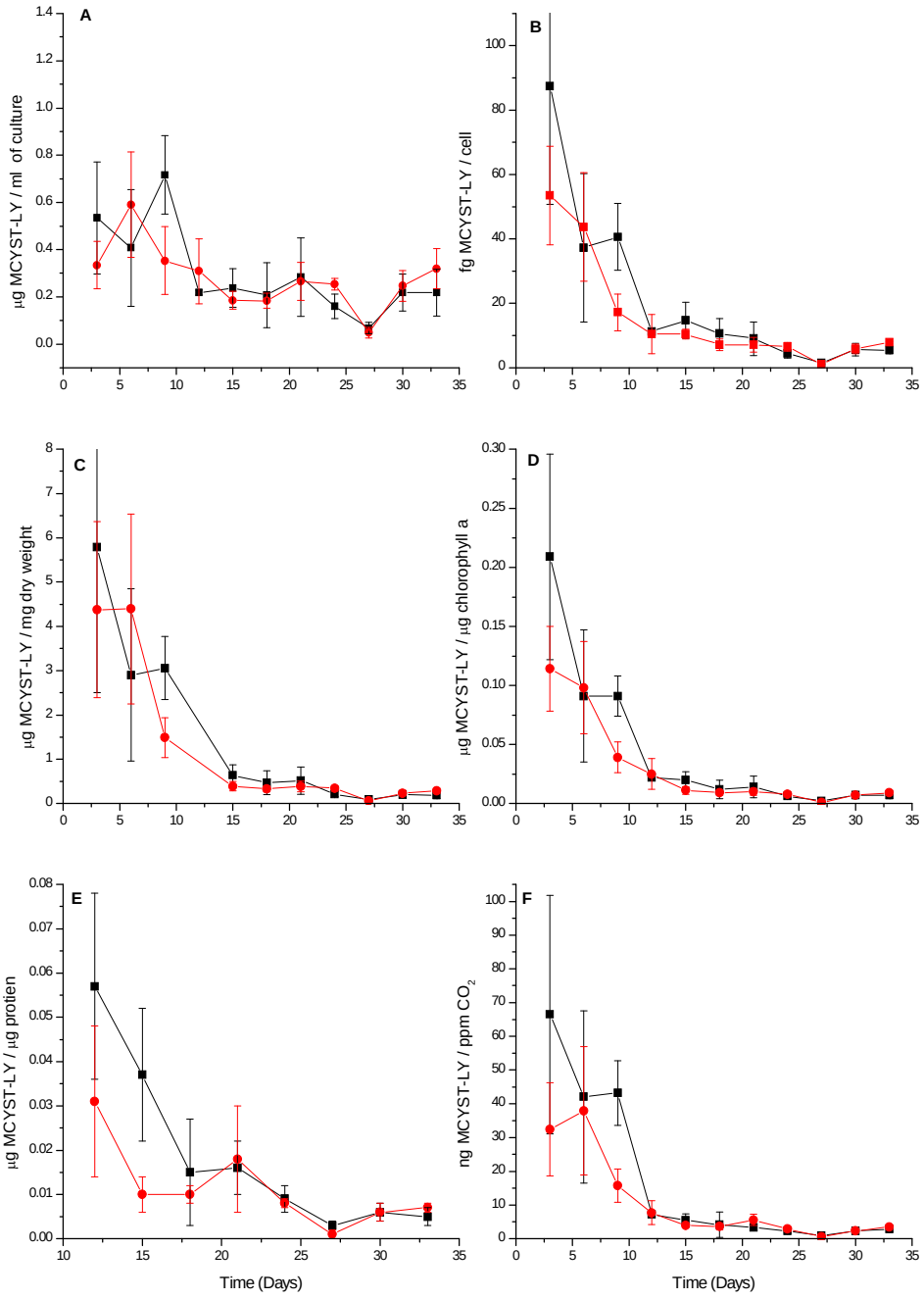

Figure 3.11
Effects of 1 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LY levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LY reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LY / ml of culture, (B) fg MCYST-LY / cell, (C) µg MCYST-LY / mg dry weight of cells, (D) µg MCYST-LY / µg chlorophyll a, (E) µg MCYST-LY / µg protein and (F) ng MCYST-LY / ppm CO_2.

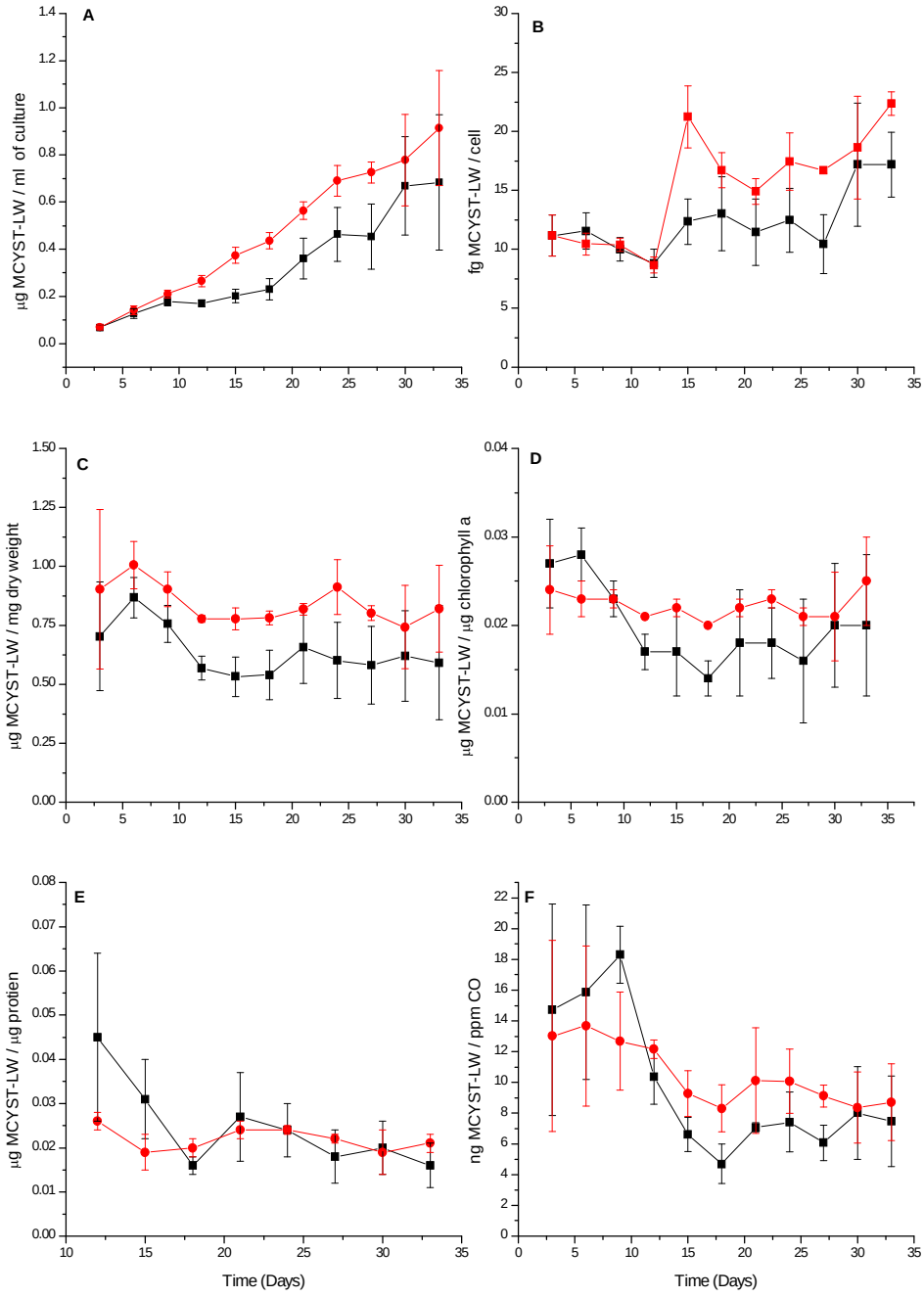

Figure 3.12
Effects of 1 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LW levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LW reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LW / ml of culture, (B) fg MCYST-LW / cell, (C) µg MCYST-LW / mg dry weight of cells, (D) µg MCYST-LW / µg chlorophyll a, (E) µg MCYST-LW / µg protein and (F) ng MCYST-LW / ppm CO_2.

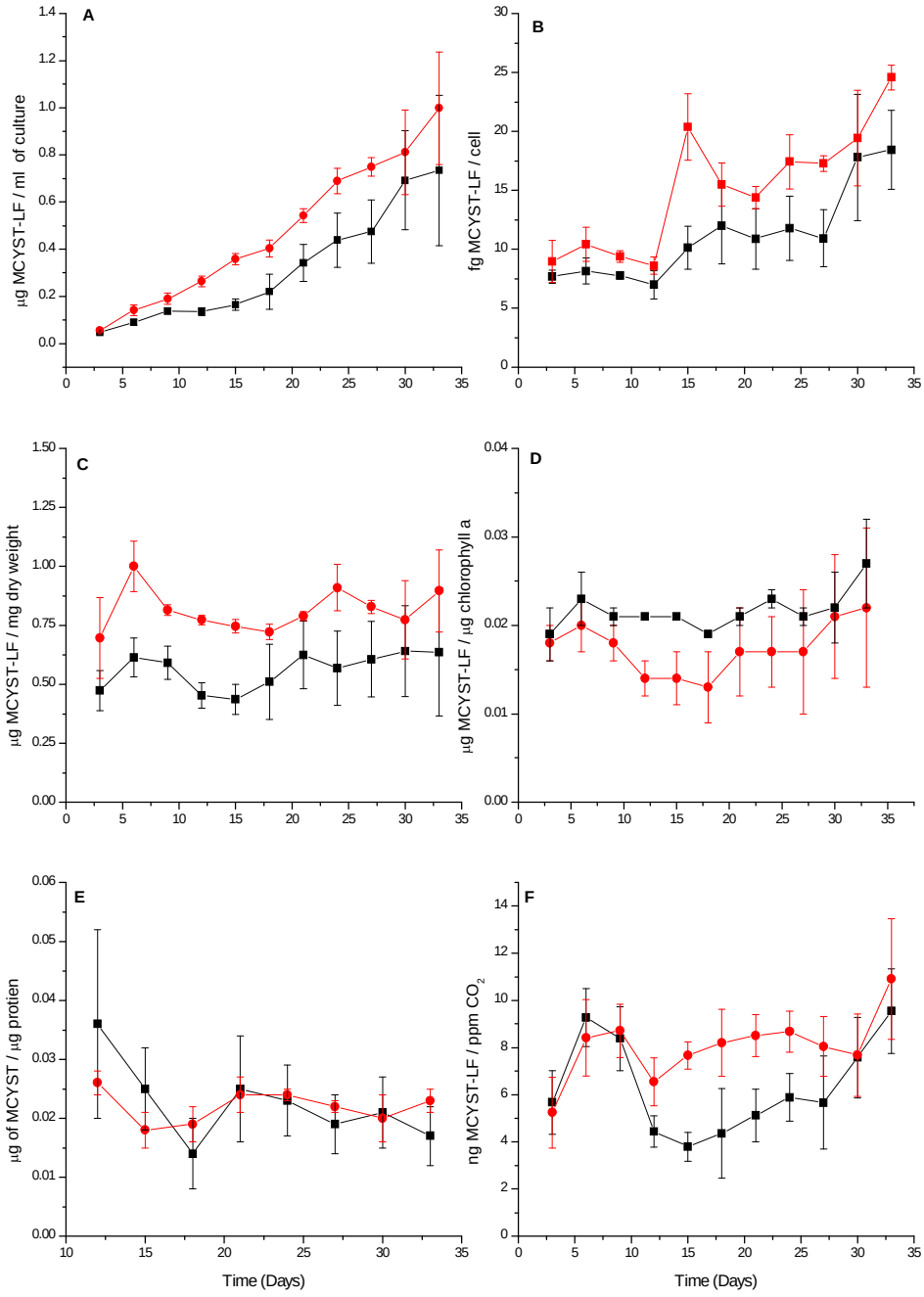

Figure 3.13
Effects of 1 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LF levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LF reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LF / ml of culture, (B) fg MCYST-LF / cell, (C) µg MCYST-LF / mg dry weight of cells, (D) µg MCYST-LF / µg chlorophyll a, (E) µg MCYST-LF / µg protein and (F) ng MCYST-LF / ppm CO_2.

On analysis of the media to determine extracellular content of MCYSTs (Figure 3.14) it was discovered that the level of MCYST-LY remained constant through out the experiment unlike the other MCYSTs present. The extracellular level of MCYST-LR, LW and LF were all found to increase as time progressed but no significant differences were observed between the cultures grown with air sparging and those which were exposed to 1 hr in every 24 hours of air enriched with 5% CO_2. Therefore it is clear that increasing the level of available inorganic carbon causes an increase in most of the MCYSTs produced by *M. aeruginosa* PCC7820 and does not increase cellular export of these compounds. It was also noted that since the introduction of standardizing the inoculation of cultures with cells in exponential phase the level of extracellular MCYSTs has been greatly reduced and the increases observed are as a result of the culture aging as opposed to cell death.

Improvements to the experimental design finally produced consistent growth in the cultures and the introduction of a manifold improved the consistency of the flow rate. Also reducing the time in which the cultures were exposed to air enriched with 5% CO_2 reduced the pH fluctuation to a minimum, ensuring that the cultures were not only able to grow but thrived in such conditions. Also, the use of a cannula and triple port Connecta reduced the possibility of contamination; decreased

time required for sampling and improved the efficiency of the experimental set up. Although this experimental set up was working well one disadvantage was that it relied on manual gas changes and therefore was very labour intensive when an experiment was run for a long period of time.

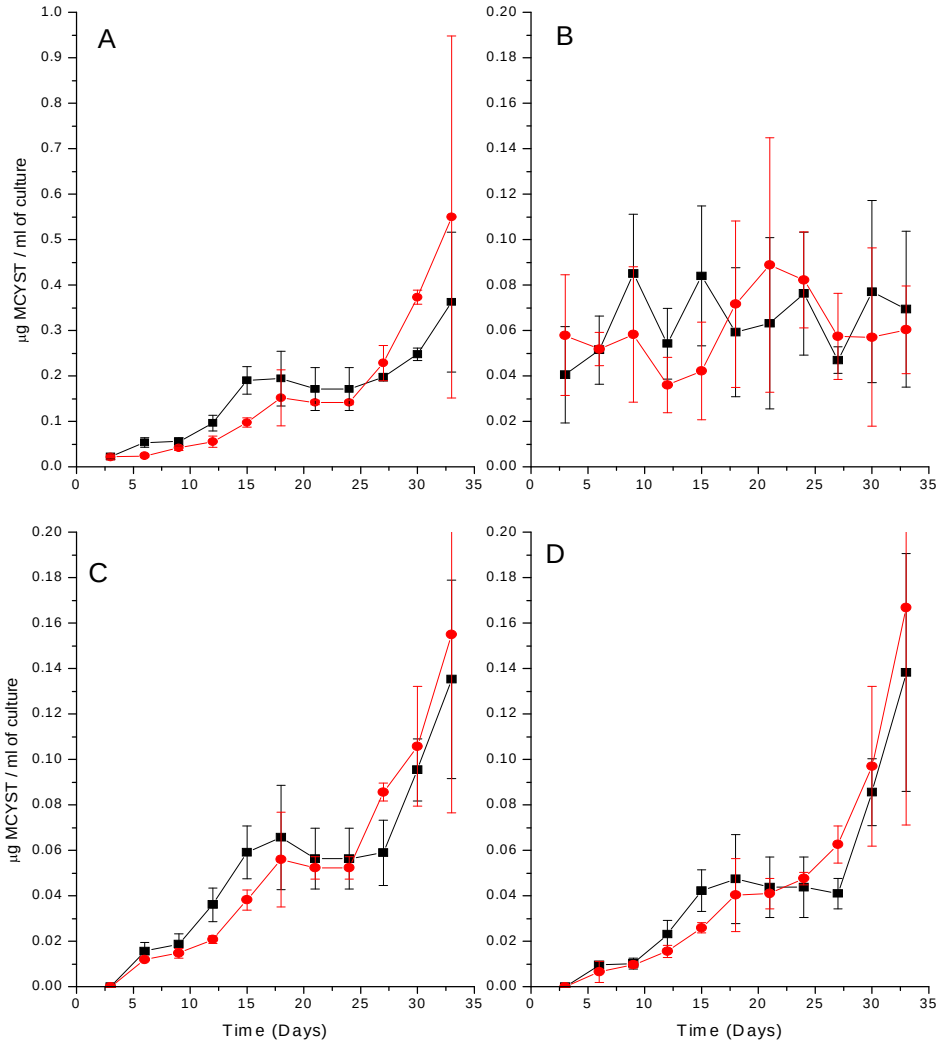

Figure 3.14
Effects of 1 hr in every 24 hours inorganic carbon (CO_2) sparging on extracellular MCYST levels in culture media with MCYST shown as µg / ml of culture for (A) MCYST-LR, (B) MCYST-LY, (C) MCYST-LW and (D) MCYST-LF Air ■, 5% CO_2 in air ●

When the total MCYST-LR levels for both intracellular and extracellular levels were compared the effect of daily 1 hr sparge with 5% CO_2 in air reduced the total MCYST-LR levels (per ml of culture) for ~ 27 days. Then the levels increased above those observed in the cultures only sparged with air (Figure 3.15a). Also when the percentages of MCYST-LR detected either intra or extracellularly in both conditions were examined they were almost identical (Figure 3.15b) with approximately 90% being retained internally under both atmospheric conditions.

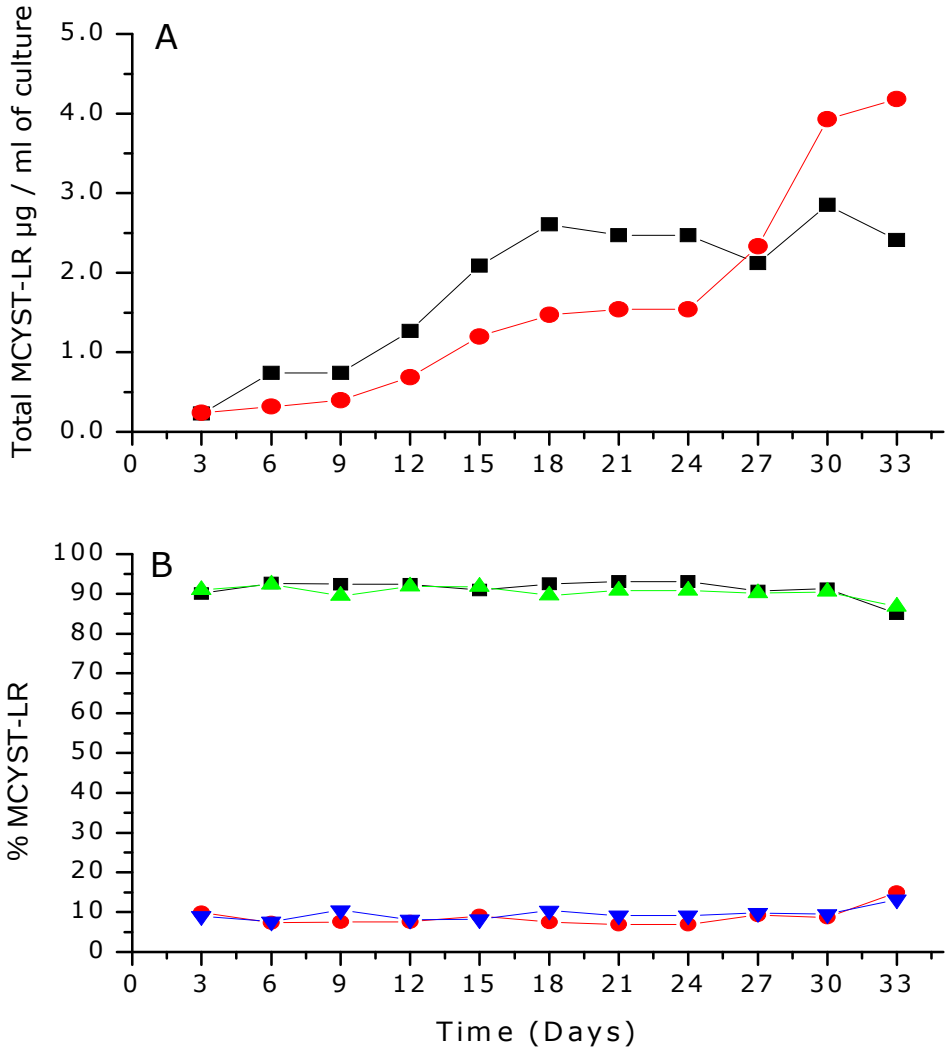

Figure 3.15
Comparison of the effects of 1 hr in every 24 hours inorganic carbon (CO_2) sparging on (A) total MCYST-LR for cultures sparged with Air ■ or 5% CO_2 in air ●.(B) % MCYST-LR detected intra (IC) and extracellularly (EC) for Air IC ■, EC ● and for 5% CO_2 in air IC ▲ and EC ▼.

3.3.5 Effect of sparging at 12 hr intervals with 5% CO_2 in air on MCYST levels using version 4

The effect on MCYST levels after growing in the presence of increased inorganic carbon through sparging with 5% CO_2 enriched air were so profound it was decided to investigate this further and introduce sparging at 12 hr intervals. In order to achieve this consistently the experimental set up of version 3 in section 3.2.4 was modified to automate the switching over of gasses for 1 hr sparging in every 12 hours to cultures (Section 3.2.56 and Figure 3.4) which were exposed to air enriched with 5% CO_2. Using this automated system cultures were able to be sparged reliably for a period of 1 hr every 12 hrs throughout the 30 days in which the experiment was carried out. Increased inorganic carbon through 2 hr of sparging per day was fond to cause no significant difference in biomass or cell numbers when compared to cultures in which no increase in inorganic carbon was supplied (Figure 3.16).

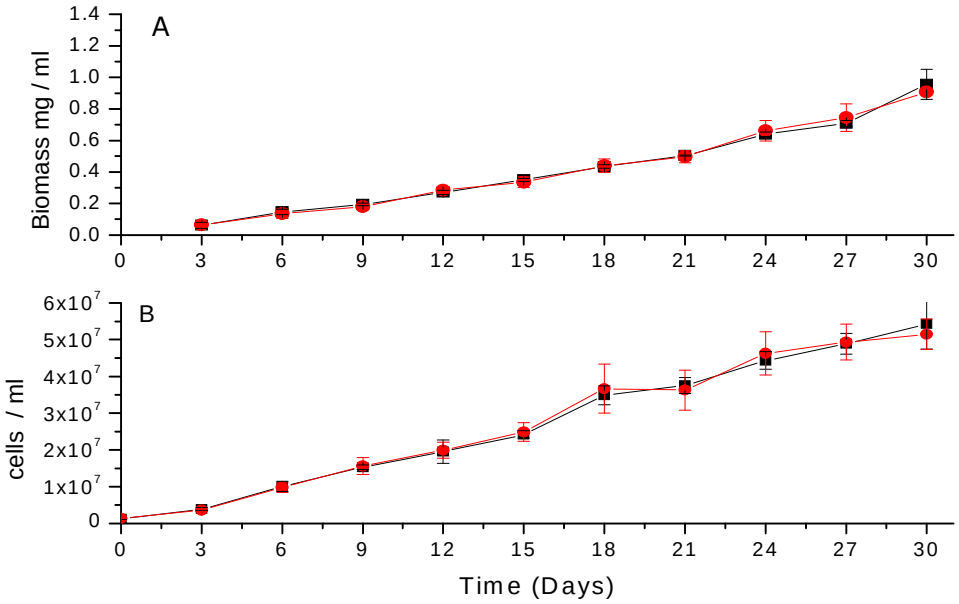

Figure 3.16
The effect on biomass (A) and cell counts (B) for cultures exposed to 2hr with 5% CO_2 enriched air over a period of 33 days. Air ■ and 5% CO_2 enriched air ●.

The intracellular MCYST-LR content was however affected as the level of MCYST-LR (Figure 3.17) was found to increase by 2 to 2.5 fold (depending on the type of analysis) under the raised inorganic carbon conditions. In all the tests carried out the standard deviation between the four replicates was also greatly reduced and provided an increase in confidence to these results. Previously exposure to only 1 hr of 5% CO_2 enriched air per day caused a decrease in MCYST-LY which conflicted with the effects observed by the other MCYST which all increased. A similar conflicting trend was again observed in the 2 hr sparged cultures

as MCYST-LY was also greatly reduced while the other three MCYSTs produced were increased (Figures 3.17 - 3.20).

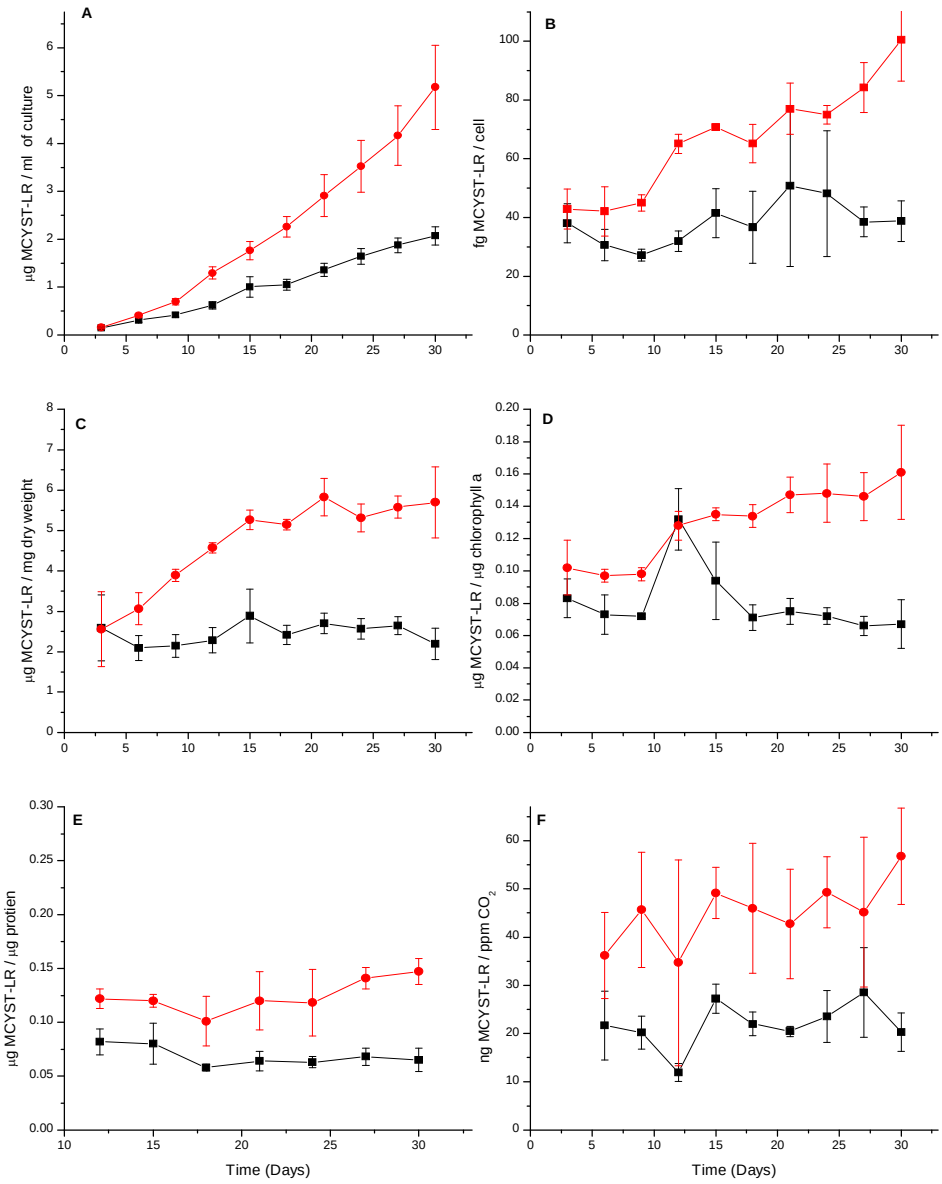

Figure 3.17
Effects of 2 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LR levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LR reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LR / ml of culture, (B) fg MCYST-LR / cell, (C) µg MCYST-LR / mg dry weight of cells, (D) µg MCYST-LR / µg chlorophyll a, (E) µg MCYST-LR / µg protein and (F) ng MCYST-LR / ppm CO_2.

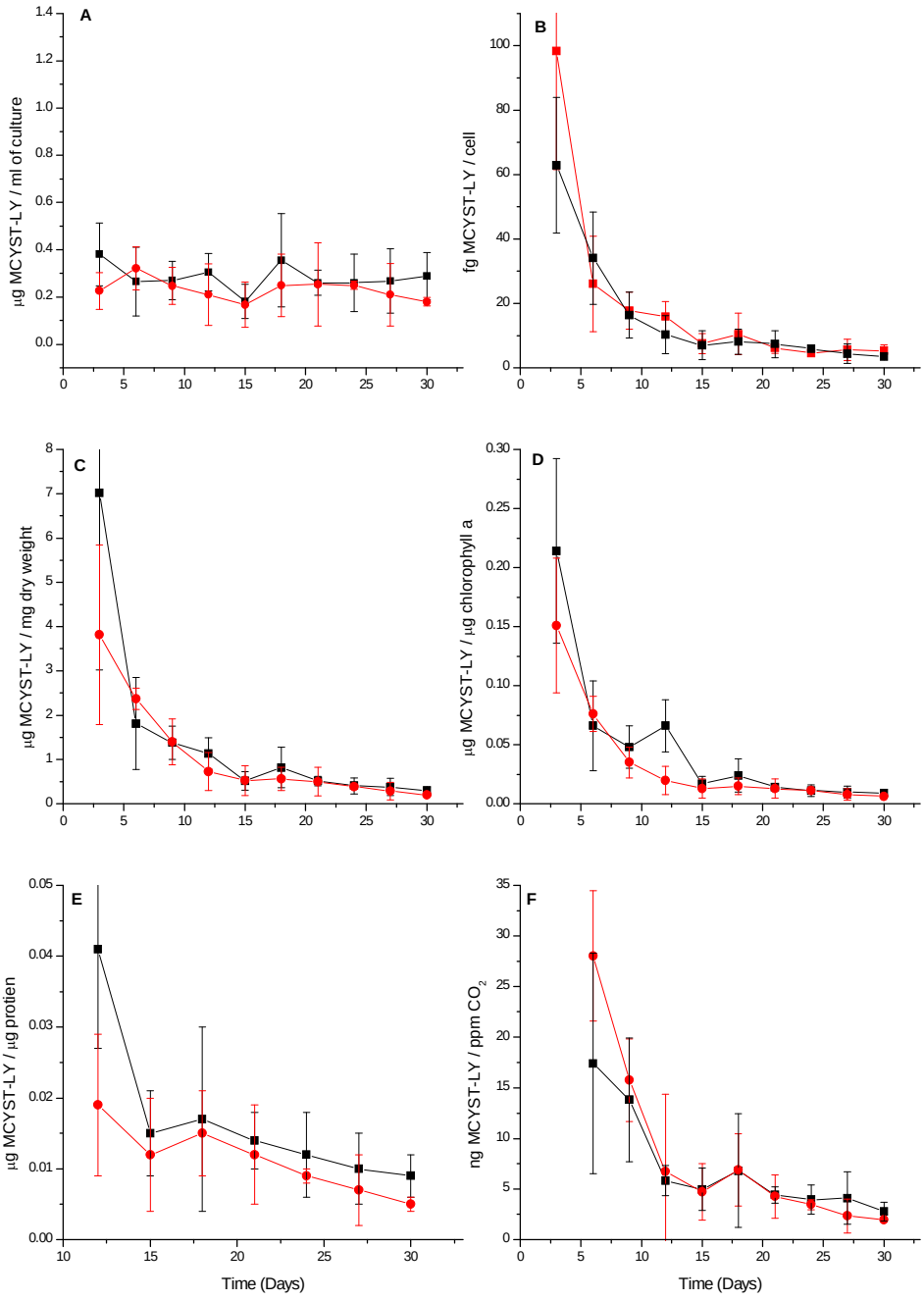

Figure 3.18
Effects of 2 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LY levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LY reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LY / ml of culture, (B) fg MCYST-LY / cell, (C) µg MCYST-LY / mg dry weight of cells, (D) µg MCYST-LY / µg chlorophyll a, (E) µg MCYST-LY / µg protein and (F) ng MCYST-LY / ppm CO_2.

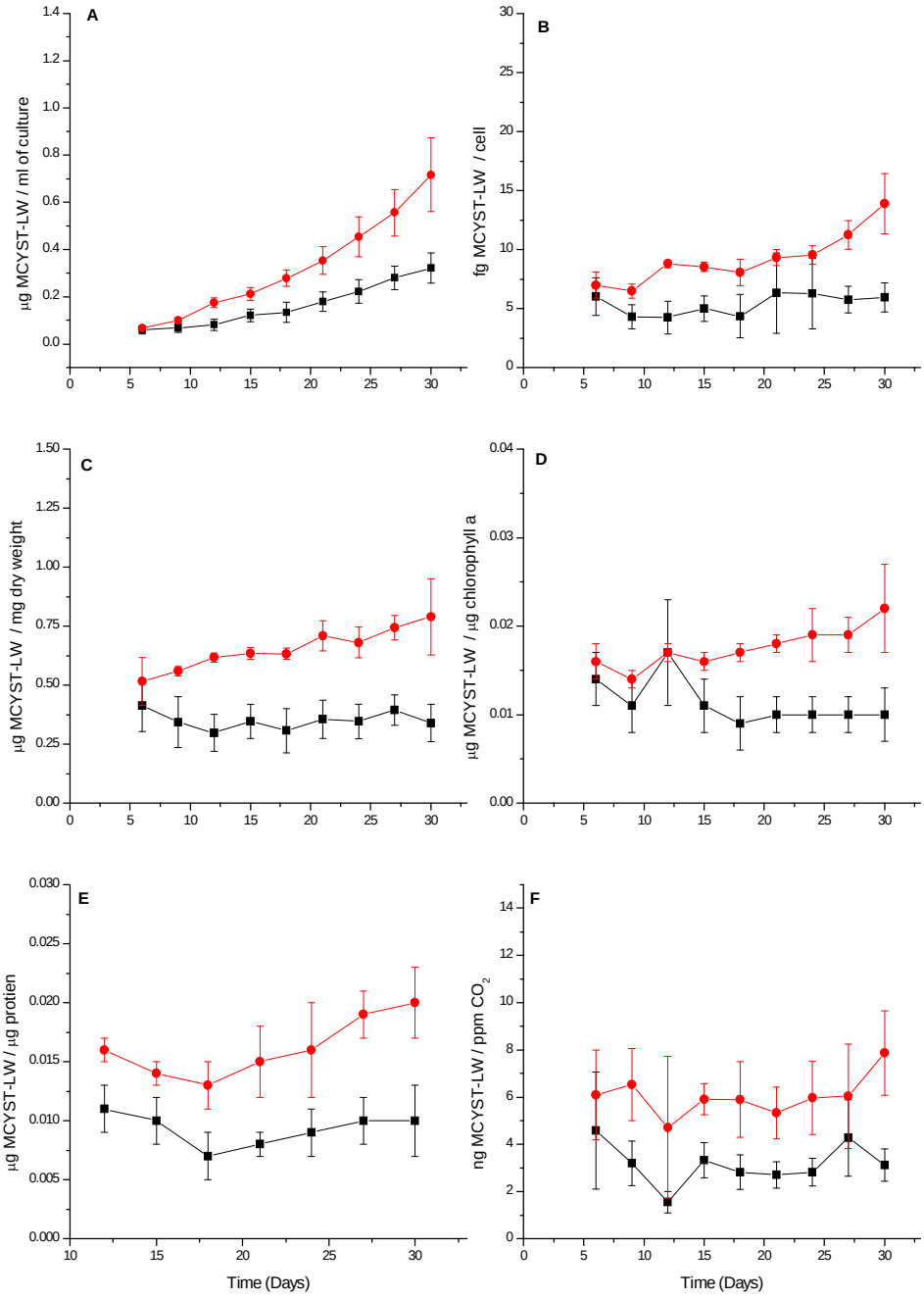

Figure 3.19
Effects of 2 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LW levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LW reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LW / ml of culture, (B) fg MCYST-LW / cell, (C) µg MCYST-LW / mg dry weight of cells, (D) µg MCYST-LW / µg chlorophyll a, (E) µg MCYST-LW / µg protein and (F) ng MCYST-LW / ppm CO_2.

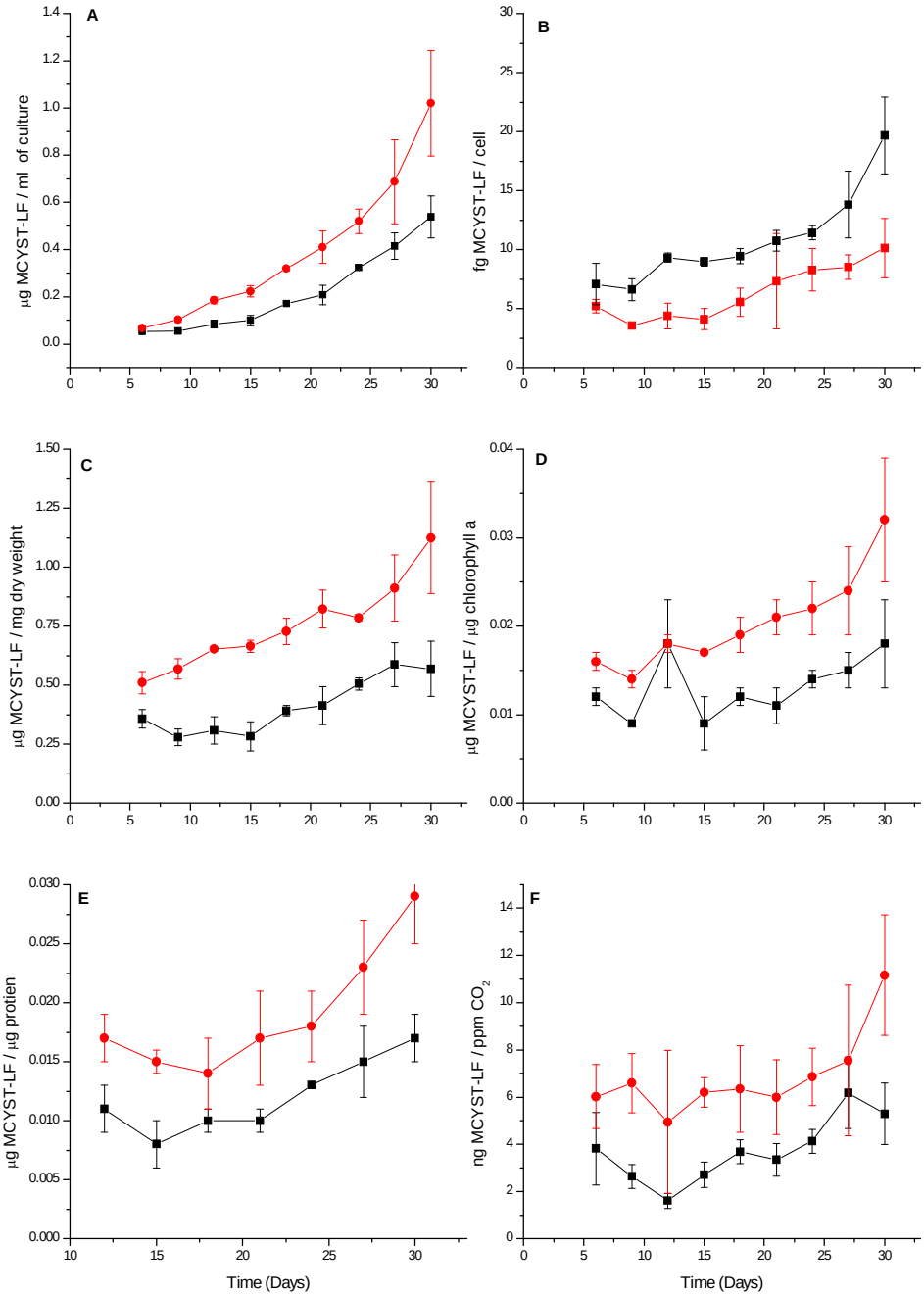

Figure 3.20
Effects of 2 hr inorganic carbon (CO_2) sparging every 24 hours on intracellular MCYST-LF levels, in cultures of *M. aeruginosa* PCC7820 over a period of 33 days with MCYST-LF reported in relation to different tests carried out. Using the following representations Air ■, 5% CO_2 in air ●,(A) µg MCYST-LF / ml of culture, (B) fg MCYST-LF / cell, (C) µg MCYST-LF / mg dry weight of cells, (D) µg MCYST-LF / µg chlorophyll a, (E) µg MCYST-LF / µg protein and (F) ng MCYST-LF / ppm CO_2.

The effect of increased inorganic carbon on the levels of MCYST-LW and LF are shown in Figures 3.19 and 3.20 and were found to be very similar to those observed for the level of MCYST-LR. These results follow the same type of trends as those observed in the 1 hr experiment, but repeated exposure every 12 hr gave a clearer indication of the effect of sparging with air enriched with 5% CO_2 compared to those sparged with only air had on cultures. As for the level of extracellular MCYSTs (Figure 3.21), they were also found to follow similar trends to those observed in the 1 hr enhanced inorganic carbon experiment, with controls and enhanced CO_2 having similar external quantities for MCYST-LR, LY , LW and LF.

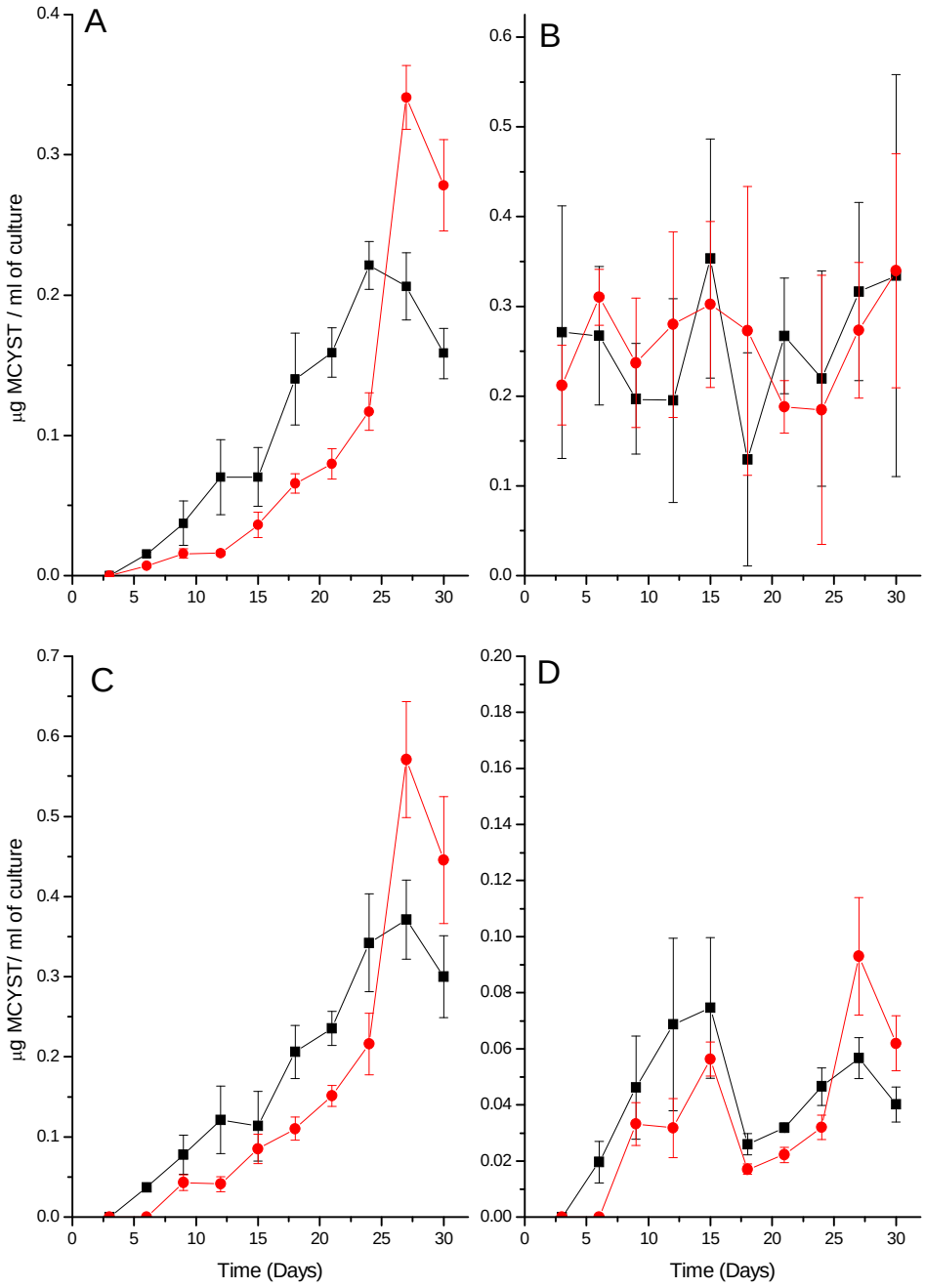

Figure 3.21
Effects of 2 hr in every 24 hours inorganic carbon (CO_2) sparging on extracellular MCYST levels in culture media with MCYST shown as µg / ml of culture for (A) MCYST-LR, (B) MCYST-LY, (C) MCYST-LW and (D) MCYST-LF Air ■, 5% CO_2 in air ●

One concern during this experiment was the effect that sparging cultures had on the pH of the media since the media does not contain a buffering agent to maintain a constant pH. Therefore samples were taken over a 24 hr period and compared to the control cultures to determine how quickly the cultures are able to recover if at all (Figure 3.22). The pH within the control samples sparged with only air, were tested after 3 days of growth and the pH has increased from 7.6 at the start to ~9.5. This was consistent over a 24 hr period however in the cultures treated with air enriched with 5 % CO2 for 1 hr it can be clearly seen that the pH immediately drops to ~6.5 during this hour and then slowly recovers over the 24 hr period to bring the pH back to ~9.5. While the pH in the cultures sparged twice a day drops to ~6.5 and then returns to a pH of ~9.0 within 12 hr before recovering once more.

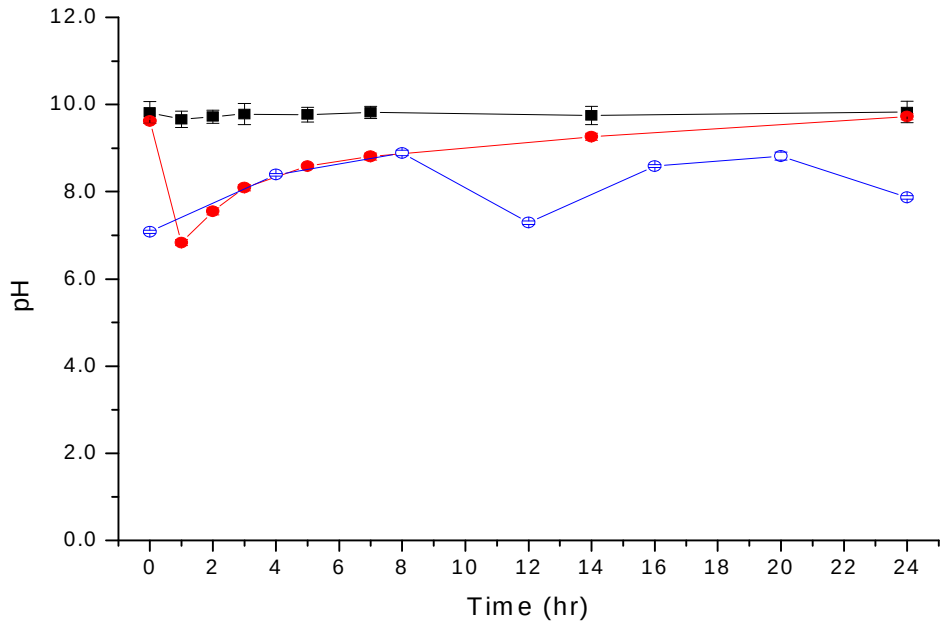

Figure 3.22
Effect on the pH of culture media over a 24 hr period of growth during CO_2 enrichment, where the following symbols were used, Air ■, 1 Hr 5% CO_2 enriched air ●. 2 Hr 5% CO_2 enriched air ○.

Although many studies have been carried out into inorganic carbon uptake (Coleman, 1991;Espie *et al.*, 1991;Miller *et al.*, 1985;Miller *et al.*, 1990;Paerl, 1983;Reuter *et al.*, 1992) none of these studies have detailed the effects of increased inorganic carbon on the media and how pH changes after increasing the level of available inorganic carbon. The work presented here shows the development of a highly standardized protocol, it has also applied an automated CO_2 enrichment culture system with improved sampling procedure. This experimental design may be applicable to many future studies into cyanobacterial growth.

This research conclusively demonstrates that elevated inorganic carbon in the form of CO_2 increases MCYST levels in all except one variant (MCYST-LY). It can be concluded that this is selectively elevated MCYST levels and doesn't just reflect a general increase in peptide / protein synthesis, as protein levels remain constant (Utkilen *et al.*, 1995). This work warrants further attention since it may indicate a role for CO_2 in MCYST regulation (apart from MCYST-LY) in the cell. However it must be noted that all MCYST research to date only represents MCYST levels at the determined sampling times this is not strictly a measure of production. Nothing is currently known about the production and in intracellular degradation rates of MCYSTs, and while methods for monitoring gene regulation (mRNA levels) do give some indication of increased synthesis results have not always matched the amounts of expressed MCYST detected (Utkilen *et al.*, 1992).

Secondly these findings may be of significance with respect to global climate change. Atmospheric CO_2 has been found to increase from c. 270 ppm CO_2 to C. 375 ppm over the last 100 years or so (IPCC, 2001) This may suggest that continual CO_2 increase may increase bloom toxicity doesn't seem to affect biomass though significantly more work is required before this conclusion can be made for certain.

Chapter 4

Biodegradation of MCYST

4.1 Introduction

For the foreseeable future cyanotoxins, especially MCYSTs will be a problem in freshwaters. It is therefore important to appreciate the persistence of these compounds in nature and possibly explain natural degradation pathways by bacteria in water treatment. Understanding these naturally occurring bacteria and their efficiency in degrading not only MCYST but also other microbial contaminants could prove beneficial as the cost of water treatment rises and demand for safe water increases. Although, (Bourne *et al.*, 1996) characterized the enzymatic pathway *Sphingomanas* sp. ACM-3962 utilises for the degradation of MCYST-LR (MW=994) where an enzyme called Microcystinase attacks the peptide MCYST-LR and cleaves the cyclic ring to form a linearised MCYST-LR (MW=1012). This linearised MCYST is then degraded further by Enzyme 2 to give a compound called Tetra-Peptide (NH_2-Adda-isoGlu-Mdha-Ala-OH) (MW=614) and finally Enzyme 3 degrades the Tetra-Peptide into undetected smaller peptides and amino acids. Many other bacteria are also capable of such degradation but have yet to be identified along with their modes of action revealed.

Water samples taken from both lochs and rivers in the Aberdeenshire and Angus area came from three locations, with considerably different

microbial histories. The river Carron is a fast flowing river unable to support cyanobacterial blooms, with seasonal changes to the water chemistry mainly as a result of run off from the surrounding farm land. Then Forfar loch (hypertrophic) a relatively small water body that has had a history of non-toxic cyanobacteria and high levels of both ammonia (2.75 mg/L) and phosphate (2.68 mg/L) depending on the time of year (Details were supplied by SEPA, Arbroath, Scotland). These levels have however been gradually decreasing over the past 10 years and are now on average below 1 mg / L (SEPA, Arbroath, Scotland), even though it has been in the path of a sewage treatment plant discharge for the past five years. The Loch is also populated by a large number of birds and numbers have been recorded as high as 900 although the average is usually between 300-400 throughout the year these include anything from black headed gulls to ducks and geese (SEPA, Arbroath, Scotland). The last water body tested is Rescobie loch (eutrophic) this loch is connected to Balgavies loch that has already been well documented for its prior history of toxic cyanobacterial blooms and where *M.aeruginosa* PCC7820 was first isolated (Phillips *et al.*, 1985). On several occasions treatment with straw bales have been used as a treatment for bloom formations, the way in which these work has been studies by (Welch *et al.*, 1990). Who discovered antibiotic production by the fungal flora or to the

release of phenolic compounds such as ferulic acid and f-coumaric acid during the decomposition of the straw walls was the mode of action. All methods for the treatment of toxic cyanobacterial blooms, inevitably result in release of toxins into the surrounding environment and therefore this chapter studied the biodegradation potential of microbial populations in waters from various locations in the Aberdeenshire area. It also tried to identify if naturally occurring microbial species capable of degrading cyanotoxins increase their tolerance to such compounds after repeated low exposure or evolve to utilize these toxic compounds as a source of carbon and energy (Bourne *et al.*, 1996). Studies by (Bourne *et al.*, 1996;Jones *et al.*, 1994;Lahti *et al.*, 1997;Rapala *et al.*, 2005) have all reported microbial degradation of MCYSTs or NOD and some species have even been identified, but most degradation pathways are yet to be fully characterised. As our climate changes and carbon levels increase or as temperatures rise, the possibility of increased cyanobacterial blooms is a major concern especially the effects on the microbial populations when toxins are released through cell lysis. Therefore understanding how natural micro-ecosystems might cope or understand the likely persistence of such compounds can only be beneficial in developing methods for the prevention or treatment of bloom formations. Currently, treating cyanobacterial blooms in large water bodies is often

difficult and expensive; therefore it has usually been limited to fresh water treatment plants and recreational areas. Environmental authorities and experts agree that a rapid and cheap water resource protection and restoration method would be highly desirable, although process of review prior to investment is required (Chorus *et al.*, 1999). The aims of this chapter were to investigate microbial degradation of a range of MCYSTs and NOD, in a variety of freshwater samples. Through the addition of either single or multiple toxins under laboratory conditions, then further research was carried on the effect pre treatment has on the rate of degradation of multiple toxins. The rate of degradation was determined by quantitative LC / MS analysis.

4.2 Methods

4.2.1 Collection of water samples from lochs and rivers

Field samples were collected from freshwaters in Aberdeenshire and Angus, the location of the sampling points are given in Table 4.1. Surface water samples were collected in sterile 1 L glass bottles and returned to the laboratory within 6 h and stored at 4°C overnight. The samples were then filtered through a metal sieve (Endecotts Ltd, London, England sieve 150 μm) to remove large particles (i.e. zooplankton and vegetation) while not removing the planktonic, natural bacteria present. The water chemistry data was supplied by the Senior Environmental Quality Officer, SEPA Aberdeen where samples were analysed on a monthly basis from 2000 to 2004, small variations were observed between months some of which could be as a result of seasonal variation.

	Rescobie Loch	Forfar Loch	Carron River
History	MCYST producing cyanobacteria (*microcystis* sp.)	Non-toxic Cyanobacteria	No previous Cyanobacteria
pH	7.5 - 9.1	6.7 - 7.6	
Ammonia mg/L	<0.005 - 0.287	0.016 - 2.75	
o-Phosphate mg/L	<0.004 - 0.153	0.002 - 2.68	
BOD mg/L	1.3 - 4.3	0.11 - 14.68	
Grid Ref No	52505159	293458	877857

Table 4.1
History and water chemistry of the fresh water sources used in the bacterial degradation of MCYSTs experiments.

4.2.2 Growth and harvesting of cyanobacteria

Cyanobacteria *Microcystis aeruginosa* PCC7820 (Pasteur Culture Collection, Paris, France), *Microcystis sp*. (Sciento, Manchester, UK) and *Nodularia spunigena* KAC66 (University of Kalmar, Sweden) were grown in BG-11 plus nitrate (8.8 mM) medium (Table 2.1). Cultures were grown in 10 L vessels for 6-8 weeks at a constant temperature of 25°C, under continuous illumination from cool white fluorescent light 20 µmol $s^{-1}m^{-2}$ and constantly sparged. The cells were then harvested by tangential flow filtration (ULTRAN® - Slice Membrane Cassette, 0.20 µm open channel pore)System then centrifuged (4000 x g) to give a wet pellet; these were stored at -20 ºC until required.

4.2.3 Purification of MCYSTs and nodularin

Hepatotoxic compounds (MCYST-LR,-LY,-LW and –LF) were extracted from the wet pellet of cyanobacterial cells using the method detailed by (Lawton *et al.*, 1994) and purified using a Biotage flash 40 system (Biotage, a Division of Dyax Corporation, Charlottesvile, VA, USA). The extracted cell contents were run through C18 flash cartridges (Biotage, a Division of Dyax Corporation, Charlottesvile, VA, USA) and then eluted into different fractions until the desired MCYSTs were obtained with a high purity determined by LC-MS. The MCYSTs were then dried by rotary evaporation (BÜCHI Labortechnik AG, Switzerland) and finally dried with nitrogen in 1 mg aliquots. These were reconstituted in Milli-Q water by washing the glass vials several times with 2 ml Milli-Q water until they were completely resuspended, the individual compounds MCYST-LR, -LF and nodularin (NOD) were made up to a final volume of 30 ml. A mixture containing MCYST-RR, -LR, -LW, -LF and NOD was reconstituted in the same way as previously described, but the final volume was 60 ml as this solution was required for inoculation with more samples. The final solutions used were mixed thoroughly and then filtered through sterile syringe filters as per the manufactures instructions (Spectrum Dynagard, 3.4 cm surface area, 0.2 μm) into sterile glass bottles ready for use.

4.2.4 MCYST and nodularin degradation by natural water samples

After filtering the river and loch samples (Section 4.2.1) 50 ml aliquots were placed in 100 ml sterile conical flasks. These flasks were divided up into groups of three and each natural water sample was subjected to six different tests. The first three flasks were used as controls for the degradation of MCYSTs so they were autoclaved at 121°C for 15 min. A mixture of sterile MCYST- RR, -LR, -LW,-LF and Nod (Section 4.2.3) was added to the control flasks when cool, to give a starting concentration of 1 μg / ml for each MCYST and nodularin.

The degradation of single compounds on their own (MCYST-LR, -LF and NOD) and the mixture (MCYST-LR, -LY, -LW, -LF and NOD) was evaluated in triplicate non-sterile water samples (1 μg / ml). Finally three flasks of non-sterile water were used to test initial levels of MCYSTs within the water sample and monitor for any natural MCYST production during the course of the experiment. All flasks were kept at 29°C and shaken at 100 rpm throughout the experiment.

4.2.5 Sampling and analysis

The degradation of MCYSTs and nodularin both individually and as a mixture were monitored over a period of thirty days with 2 ml samples being removed under sterile conditions every three days. These were then frozen immediately, freeze dried, then reconstituted in 200 µl of 80:20 methanol: water and centrifuged (to remove any undissolved solids) for 10 min before analysis by LC-MS. Analysis of MCYSTs and nodularin was performed using a modification of the method by (Lawton *et al.*, 1994) high performance liquid chromatography mass spectrometry (LC-MS) was carried out using a Waters system consisting of a (Waters 2690 Separation Module). Detection was carried out by a waters mass spectrometer (Waters Micromass® ZQ^{TM}) and photodiode-array (PDA) model 996. Separation of nodularin and MCYSTs was carried out using a Waters symmetry C_{18} columns (150 x 2.1 mm, particle size 5 µm). Data was collected using mass spectrometry manager software (Masslynx v3.5). Mobile phases were A: Milli-Q water with 0.05% trifluoroacetic acid and B: acetonitrile with 0.05% trifluoroacetic acid (TFA). The mobile phases were pumped at a flow rate of 0.3 ml / min using the gradient shown in (Table 4.2); with internal degassing of the mobile phase by the solvent delivery pump.

Mobile Phase	Time (min)					
	0	25	26	28	30	35
Water 0.05% TFA	95%	40%	0%	0%	95%	95%
Acetonitrile 0.05% TFA	5%	60%	100%	100%	5%	5%

Table 4.2
HPLC Gradient for MCYST and nodularin analysis over a period of 35 min with the proportion of mobile phase expressed as a percent.

The column was maintained at 40°C, MCYST-LR standards were injected (25 μl) to give 1 μg column loading and 25 μl injections were used for all samples. Analysis was carried out using ES^+ ionization mode with cone voltage 70.00, 72.65 V with cone gas flow 104 L/hr and the desolvation gas flow 363 L/hr. The PDA detector was set to acquire data from 200 to 300 nm with resolution of 1.2 nm and the chromatograms monitored at 238 nm. A diluent (blank) and MCYST standard were tested prior to sample analysis in order to check the system performance. When processing the data the spectrum for each peak was compared with the mass for known MCYSTs and nodularins. Also the total ion chromatography (TIC), masses and spectra were studied for any unknown peaks to determine if they were breakdown products from the MCYSTs or NOD samples.

4.2.6 MCYST and nodularin degradation by pre-conditioned natural water

The water samples from lochs and rivers exposed to different MCYSTs and NOD in the initial experiment (Section 4.2.4) were further used to test if degradation rates can be affected by previous laboratory exposure to MCYST-LR,-LF, NOD or a toxin mixture (MCYST-RR, -LR, -LW, -LF / NOD). The water samples previously exposed to 1 µg / ml of MCYST and NOD for 30 days were re-exposed to the MCYST and NOD mixture (concentration 50 µg / ml) was added to increase the concentration to ~ 2.5 µg / ml in each flask. After 24 hr the first sample was removed, sampling continued at 3 day intervals for a period of 15 days in order to determine the rate of degradation measured as in section 4.2.5.

4.3 Results and discussion

4.3.1 Biodegradation of cyanobacterial hepatotoxins by natural water samples

Initially all the waters collected were analysed by HPLC to ensure that no MCYSTs or nodularins were present prior to experiments. This confirmed that only the toxins added or their breakdown products would be present in test samples (Data not included). The experiments conducted identified that the microbial population present in loch Rescobie water was capable of degrading different MCYSTs and NOD to undetectable levels over a period of 24 days. The rates of degradation were found to vary depending on the particular toxin present, for MCYST-LR the first signs of degradation were observed after 6 days and after 12 days no MCYST-LR was detected (Figure 4.1). A similar trend was also observed when either MCYST-LF or nodularin were present but the time required to reach 100% degradation was longer by 3 and 6 days respectively, when compared to MCYST-LR degradation (Figure 4.1). In the presence of the MCYST-RR, -LR, -LW, -LF / NOD mixture the rates of degradation compared to individual toxins was significantly decreased with MCYST-LR taking almost twice as long to reach almost 100% degradation (Figure 4.1). MCYST-LR did however show more degradation in the early stages in comparison to

MCYST-RR, MCYST-LW, MCYST-LF and NOD. However although the rate of degradation for MCYST-RR, MCYST-LW, MCYST-LF and NOD was slower initially they all followed a very similar trend and 24 days was required for complete degradation.

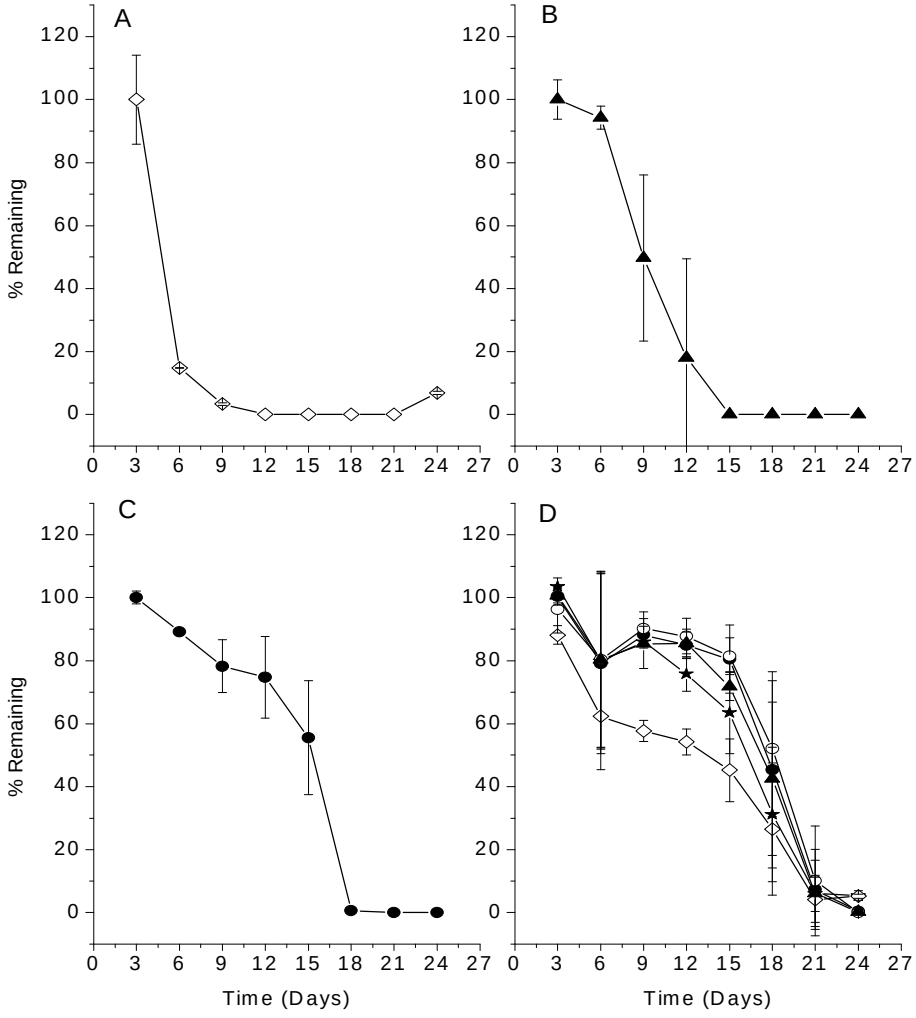

Figure 4.1
Degradation in Loch Rescobie water **A**:- MCYST-LR (1μg / ml), **B**:**-** MCYST-LF (1μg / ml), **C**:- nodularin (1μg / ml), **D**:- toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) each at a concentration of (1μg / ml).

The microbial population in loch Forfar water behaved very differently to that observed in the Rescobie waters, with the rate of degradation for the individual compounds (MCYST-LR, MCYST-LF and NOD) (Figure 4.2 A, B and C) being much slower that that observed for the same Rescobie samples (Figure 4.1 A, B and C).The results clearly indicate that the time required to reach total degradation in Loch Forfar water was increased by 6 days for both MCYST-LR and –LF but NOD never reached complete degradation. However, in the presence of MCYST/NOD mixture (Figure 4.2 D) the rate of degradation was much faster than that observed for individual samples (Figure 4.2 A, B and C). The five compounds within the mixture were not only 100% degraded within 15 days but they all showed a very similar trend to their rates of degradation (Figure 4.2 D). The degradation was also quicker and more efficient than that observed in the Rescobie water and mixture sample (Figure 4.1 D) by ~6 days.

The river microbial population which has no previous exposure to MCYSTs has slower degradation rates for the some of the variants. While the rate of degradation for MCYST-LR (Figure 4.3 A) remained relatively quick (100 % in 21 days) the degradation of MCYST-LF (Figure 4.3B) was considerably slower (100 % > 21 days) and no degradation of nodularin was observed (Figure 4.3C). The MCYST/NOD mixture showed no signs of degradation for MCYST-RR, -LF and NOD,

but MCYST-LR,-LW did have signs of degradation although it was not conclusive as the errors are rather high and the cause was unable to be determined.

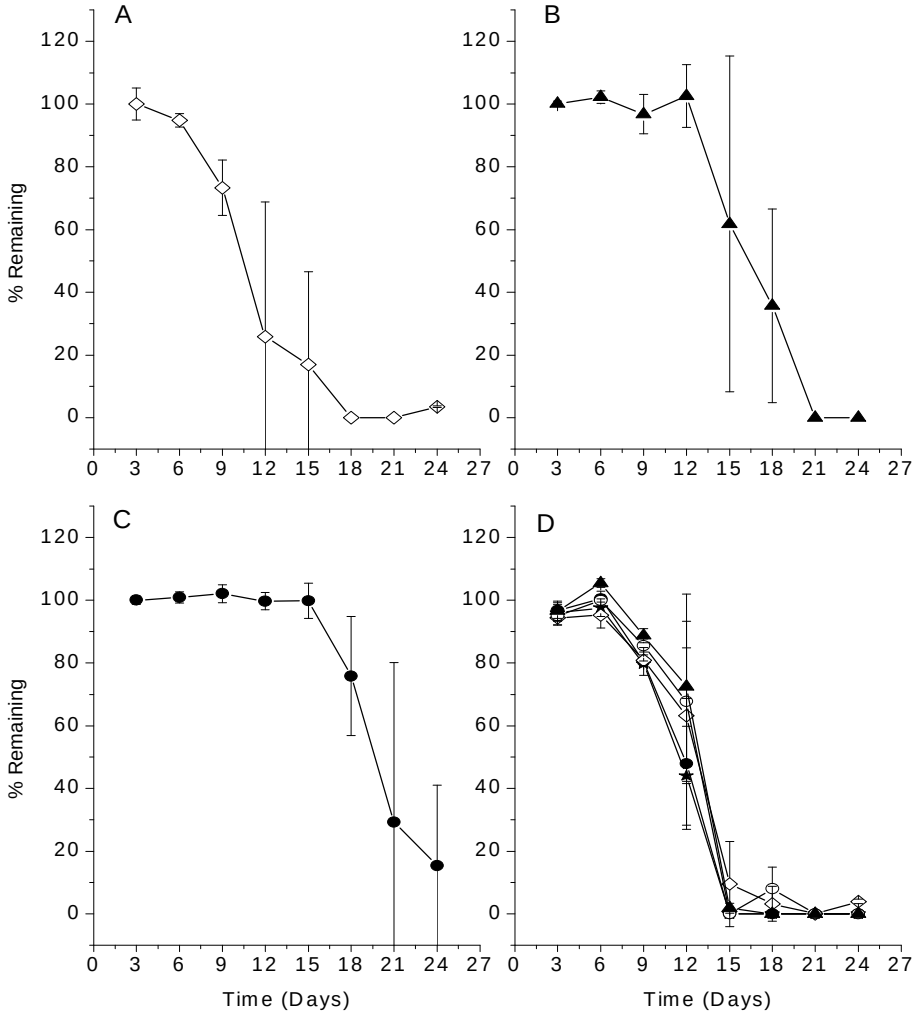

Figure 4.2
Degradation in Loch Forfar water **A**:- MCYST-LR (1μg / ml), **B**:**-** MCYST-LF (1μg / ml), **C**:- nodularin (1μg / ml), **D**:- toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) each at a concentration of (1μg / ml).

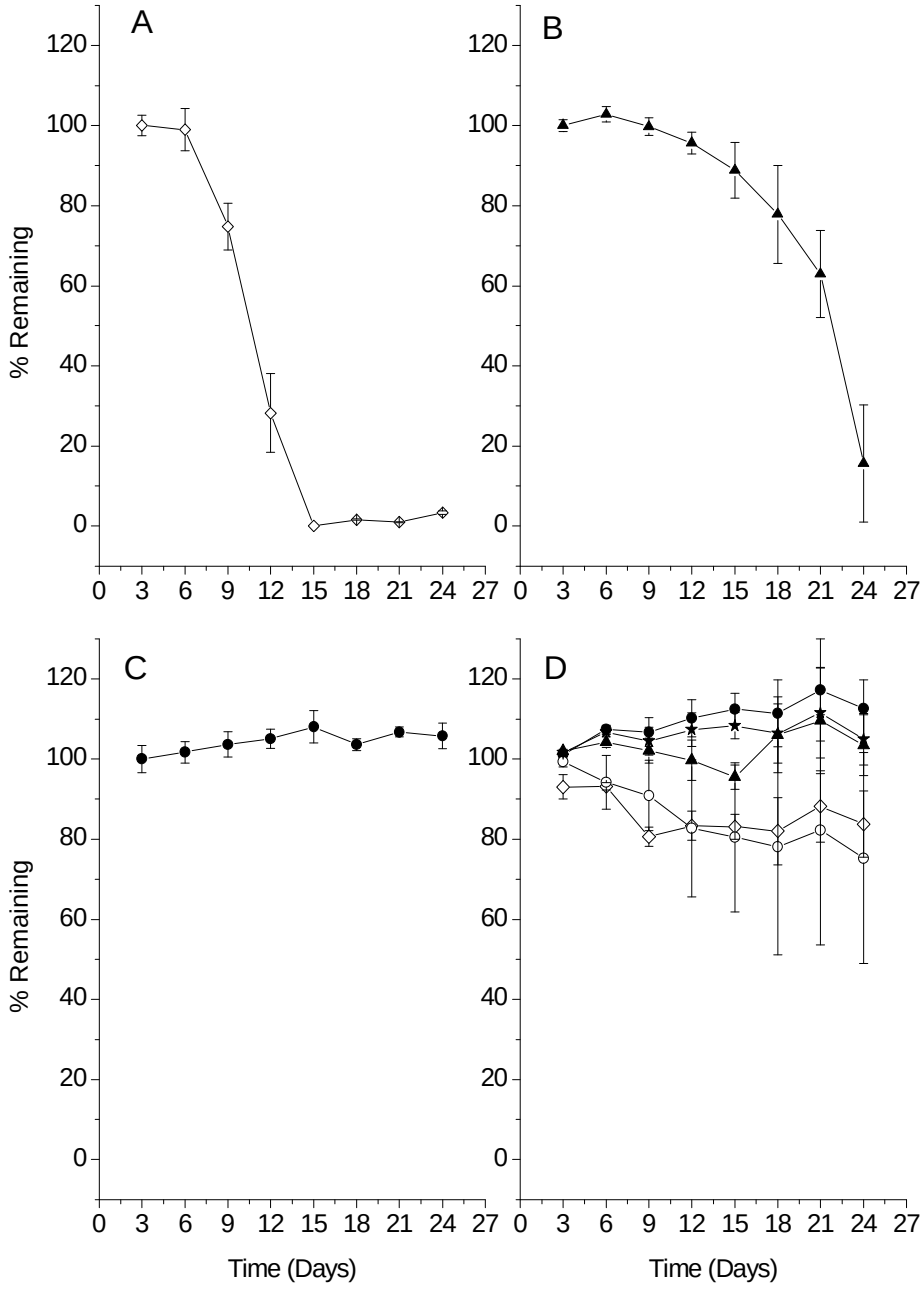

Figure 4.3
Degradation in river Carron water **A**:- MCYST-LR (1μg / ml), **B**:**-** MCYST-LF (1μg / ml), **C**:- nodularin (1μg / ml), **D**:- toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) each at a concentration of (1μg / ml).

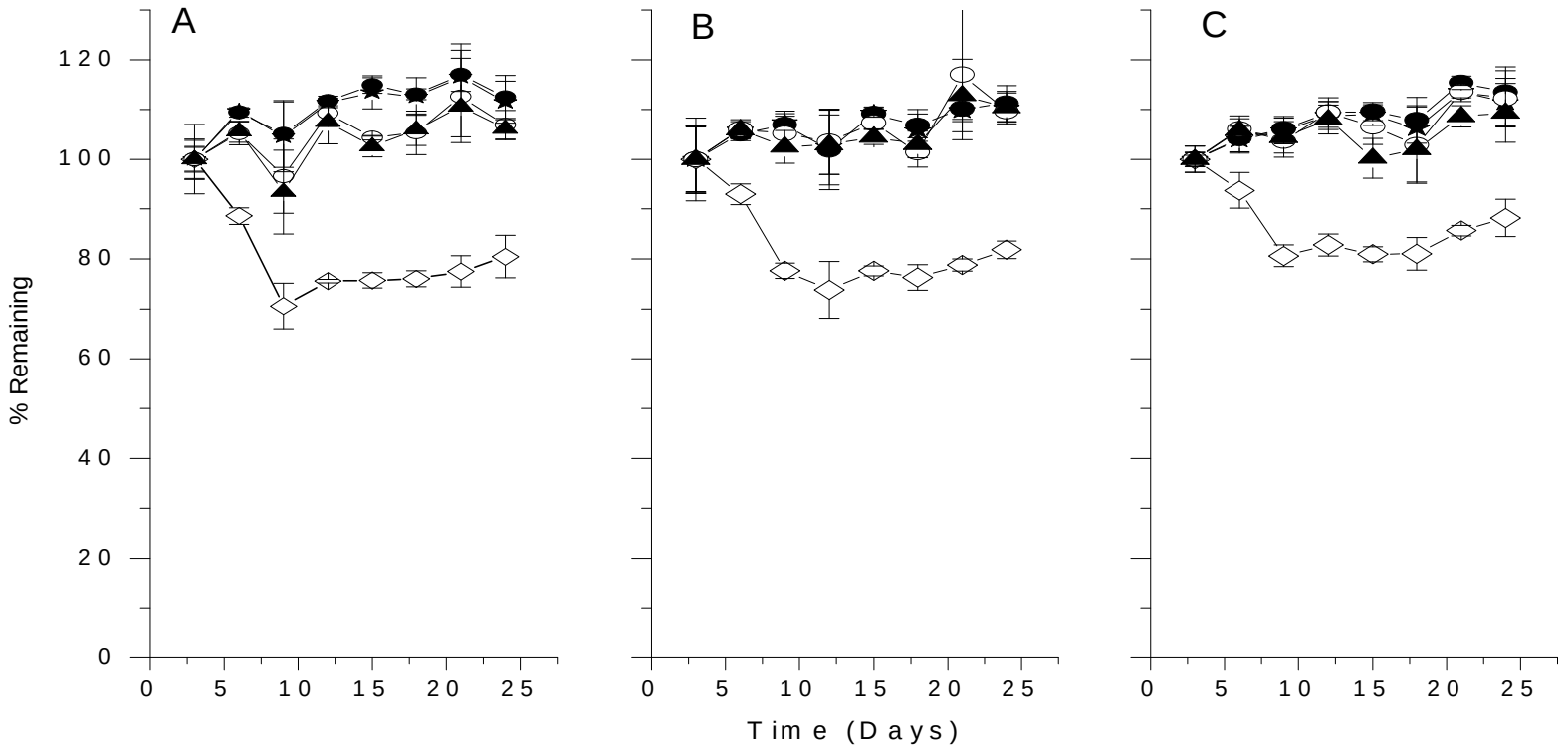

Figure 4.4
Degradation of toxin mixture in sterile controls for A- Loch Rescobie, B- Loch Forfar and C- River Carron.
Toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) each at a concentration of (1μg / ml).

In the sterile control samples (Figure 4.4) it is clear that no degradation occurred since the concentration of MCYSTs and nodularin remained at the starting concentration throughout the incubation period. A reduction in the amount of MCYST-LR recovered was observed in the sterilised samples; with levels dropping to ~70% within the first 9 days and no further reduction was observed after 9 days. This reduction in MCYST-LR could not be explained because if degradation was occurring further reductions would have been observed after 9 days. Also increases above the 100% were probably due to evaporation of liquid during the duration of the experiment.

4.3.2 Biodegradation of hepatotoxins by laboratory pre-exposed natural water samples

The water samples from loch Rescobie exposed either to; MCYST-LR, MCYST-LF, NOD or MCYST/NOD mixture (Section 4.2.4 and results in Figure 4.1), were all able to degrade a further dose of both MCYSTs and nodularin either separately or as a mixture. After the further addition of MCYST/NOD mixture to all the pre-exposed water samples the rate of degradation was monitored over a period of 15 days (Figure 4.5). When examining the effect of samples previously exposed to MCYST-LR in (Figure 4.1 A) after the addition of MCYST/NOD mixture (Figure 4.5A) it might have been expected that MCYST-LR would have been degraded faster than the other compounds present. Surprisingly,

though MCYST-RR was the only compound found to be degraded at a faster rate than the other MCYSTs/NOD present. Only 3% of MCYST-RR remained while 50-70 % of the other components still remained after 15 days of incubation. In the other samples pretreated with MCYST-LF, NOD and mixture a similar effect was also observed with rapid degradation of MCYST-RR while the other compounds remained present at much higher levels (Figure 4.5 B, C and D). The remaining MCYST/NOD compounds all were degraded at very similar rates with no significant differences observed in all four of the pretreated water samples. Although the rate of degradation was faster in both the NOD and mixture pretreatment samples respectively, with fewer compounds being degraded below 60% in the MCYST-LR and -LF pretreated samples. When comparing the rate of degradation for the initial sample of MCYST/NOD mixture (Figure 4.1D) to that of MCYST/NOD mixture after pretreatment it was observed that prior exposure results in faster degradation (Figure 4.5 D), a comparison of the rates of degradation after 15 days are detailed in Table 4.3.

Initial Water Treatment	Toxin % Remaining at 15 Days									
	MCYST-RR		Nod		MCYST-LR		MCYST-LW		MCYST-LF	
	I/T	P/T	I/T	P/T	I/T	P/T	I/T	P/T	I/T	P/T
Rescobie LR	•	3	•	57	0	47	•	67	•	63
Rescobie LF	•	23	•	70	•	90	•	46	0	72
Rescobie NOD	•	9	56	36	•	51	•	57	•	46
Rescobie Mix	64	9	81	30	45	21	81	32	71	17
Forfar LR	•	0	•	0	17	44	•	0	•	0
Forfar LF	•	0	•	0	•	2	•	0	61	0
Forfar NOD	•	1	100	0	•	3	•	0	•	0
Forfar Mix	0	3	0	0	10	4	0	0	2	1
Carron LR	•	77	•	111	81	120	•	100	•	111
Carron LF	•	55	•	100	•	103	•	88	100	91
Carron NOD	•	100	110	113	•	118	•	106	•	108
Carron Mix	108	118	112	119	83	121	81	114	96	116

Table 4.3
Comparison of % remaining after 15 days for water samples initially treated (I/T) with MCYST-LR, MCYST-LF, NOD or Mixture containing (MCYST-RR, NOD, MCYST-LR, MCYST-LW and MCYST-LF). Then to all of the initial treatment samples a further aliquot of mixture was added to give pre treated (P/T) samples containing all 5 toxins detailed above. (• Not present in initial treatment)

Water previously collected from the Forfar loch and treated with MCYST/NOD both separately and as a mixture was found to be able to degrade MCYST/NOD compounds within 15 to 24 days (Figure 4.2).

The rates of degradation of a second MCYST/NOD mixture by the pretreated water samples however increased dramatically and the majority of the compounds were completely degraded between 6 to 15 days (Figure 4.6). The most efficient and consistent degradation through out all the compounds was observed in (Figure 4.6 D) the sample previously exposed to MCYST/NOD mixture.

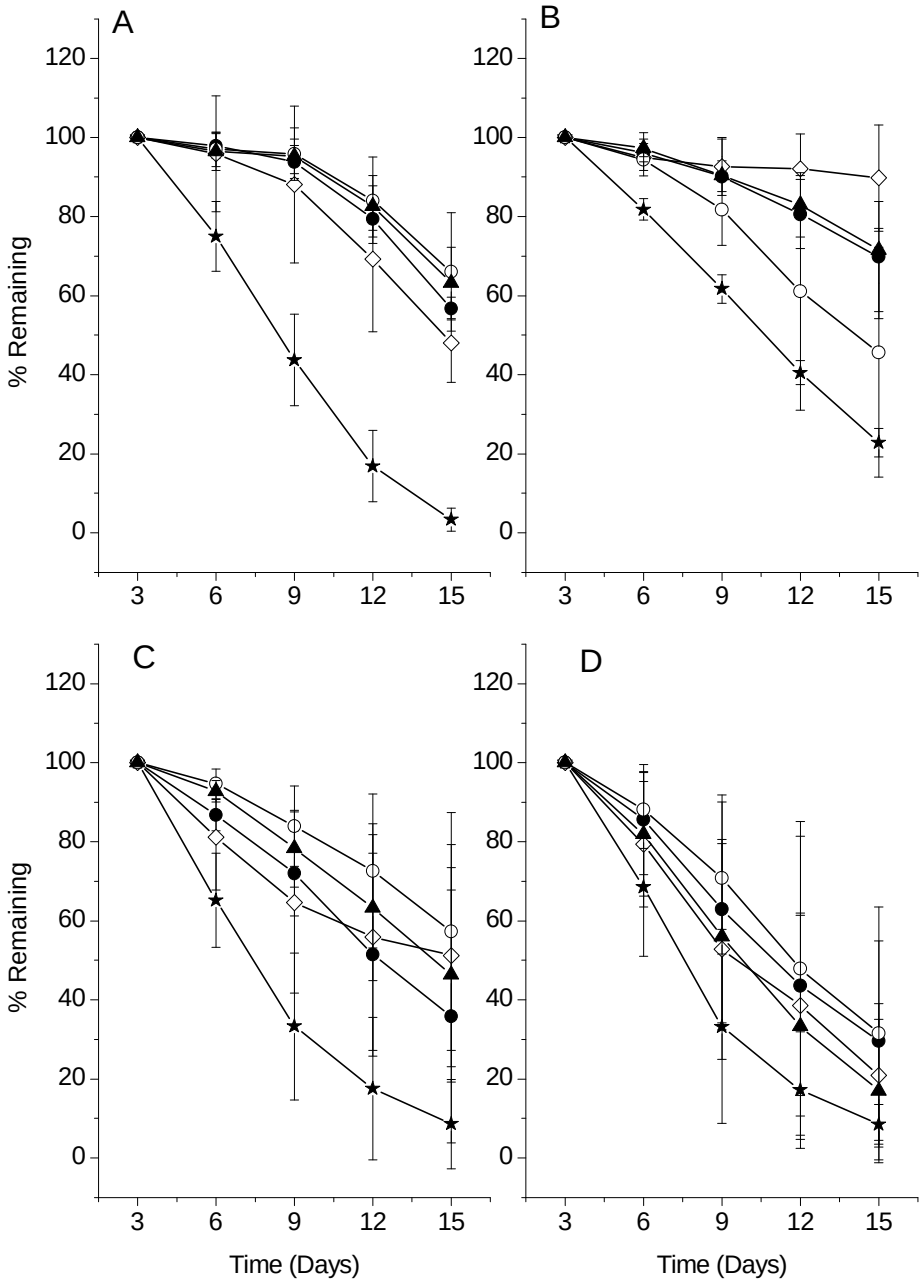

Figure 4.5
Degradation of individual MCYSTs or NOD toxin mixture by loch Rescobie water after prior laboratory exposure to **A**:- MCYST-LR (1μg / ml), **B**:**-** MCYST-LF (1μg / ml), **C**:- nodularin (1μg / ml), **D**:- toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) giving each at a concentration of (2.5 μg / ml).

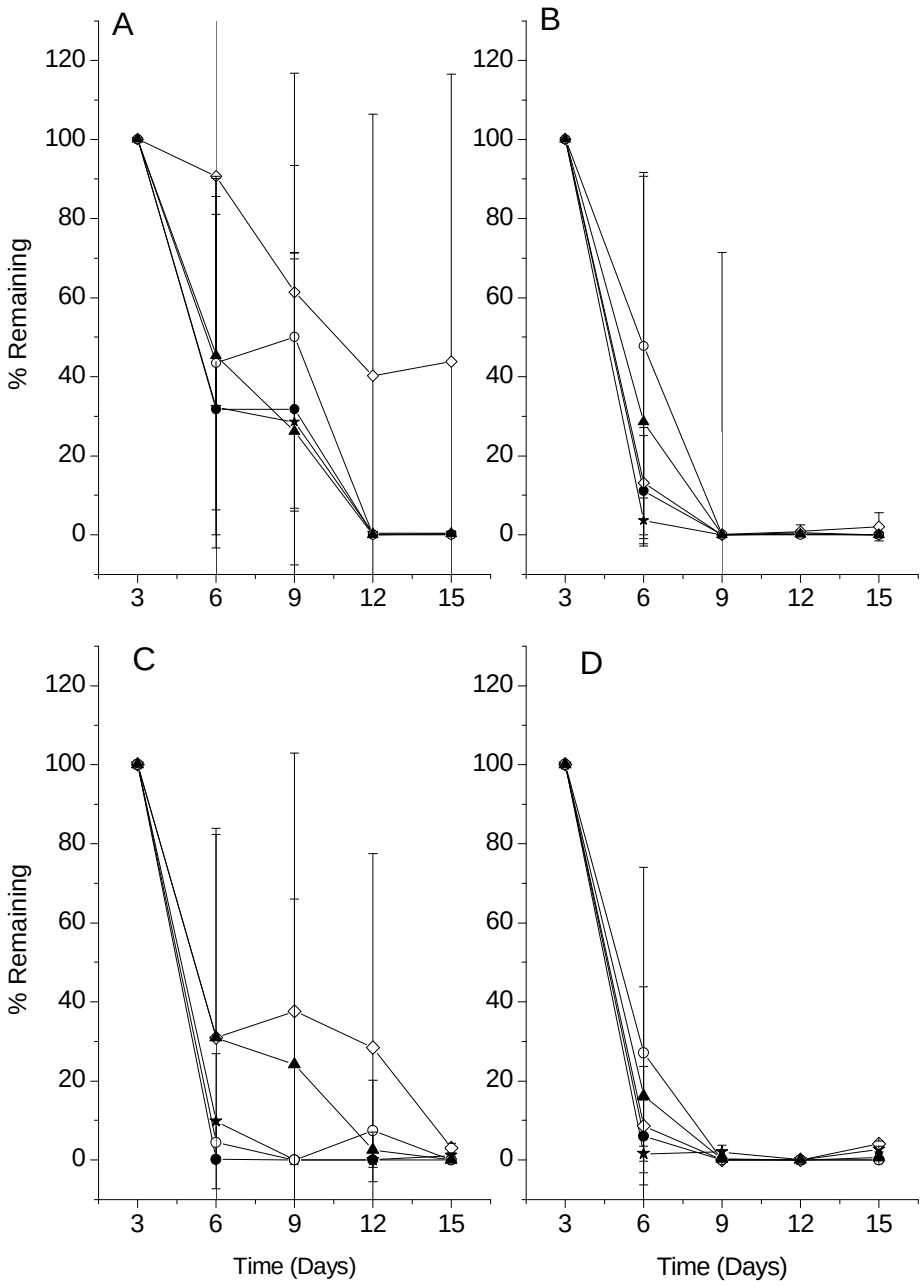

Figure 4.6
Degradation of individual MCYSTs or NOD toxin mixture by loch F water after prior laboratory exposure to **A**:- MCYST-LR (1μg / ml), **B**:**-** MCYST-LF (1μg / ml), **C**:- nodularin (1μg / ml), **D**:- toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) giving each at a concentration of (2.5 μg / ml).

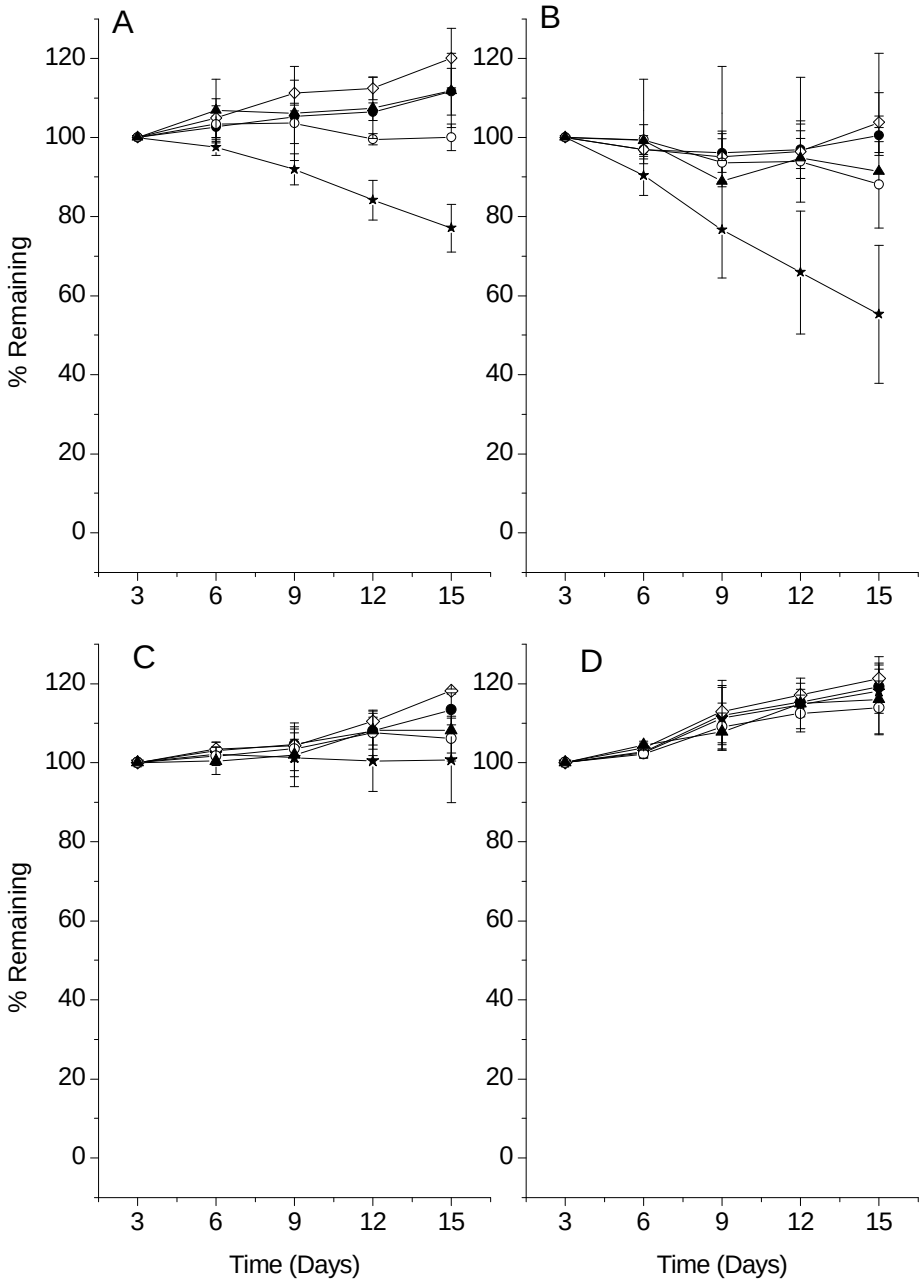

Figure 4.7
Degradation of individual MCYSTs or NOD toxin mixture by River Carron water after prior laboratory exposure to **A**:- MCYST-LR (1μg / ml), **B**:**-** MCYST-LF (1μg / ml), **C**:- nodularin (1μg / ml), **D**:- toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) giving each at a concentration of (2.5 μg / ml).

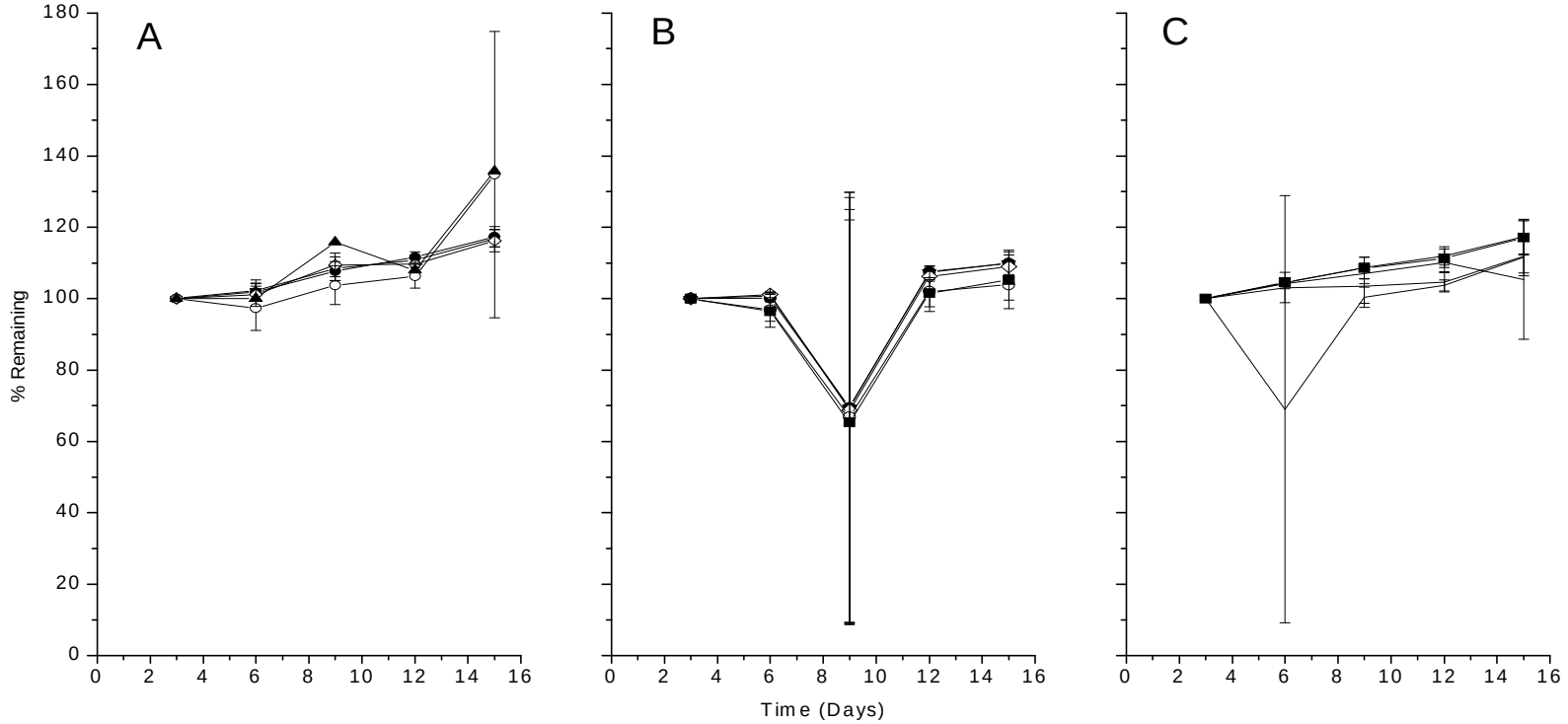

Figure 4.8
Degradation of individual MCYSTs or NOD toxin mixture in sterile controls for A- Loch Rescobie, B- Loch Forfar and C- River Carron after laboratory exposure. Toxin mixture (MCYST-RR ★, MCYST-LR ◇, MCYST-LW ○, MCYST-LF ▲ and nodularin ●) each at a concentration of (2.5 μg / ml).

Although, pretreatment with MCYST-LF shows similarly fast rates with 100% degradation of all the components within 9 days (Figure 4.6 B). It is however very difficult to determine if a particular type of pretreatment favoured the rate of degradation for any particular MCYST/NOD above any others present, as the rates seem very similar. Also one observation that can be clearly made between the initial samples and the same samples after pretreatment is that the populations of micro-organisms present in the Forfar water prefer a mixture of MCYST/NOD to individual compounds and that pretreatment increases their ability to degrade such compounds and in some cases the time required to reach complete degradation of all MCYST/NOD mixture is almost half. This may be as a result of an up regulation in the pathway for the degradation of such compounds as a result of the prior exposure to compounds such as MCYSTs.

River Carron water samples pre-exposed to MCYST/NOD both separately and as a mixture behave very differently from the initial samples as previous degradation observed (Figure 4.3 A and B) did not occur when the further MCYSTs / NOD mixture was added (Figure 4.7). No significant degradation was observed in all the pretreated water samples and only MCYST-RR showed some signs of degradation with 77 and 55% remaining after 15 days in pretreated MCYST-LR and MCYST-LF

water (Figure 4.7 A, B). There was however no degradation observed in the samples pretreated with NOD and MCYST/NOD mixture, suggesting that prior exposure is only beneficial for some microorganism populations (Figure 4.7).

When MCYST/NOD mixture was added to all the sterile controls that had previous exposure to MCYST/NOD both separately and as a mixture, no changes were observed as expected over the 15 day period of testing (Figure 4.8). Figure 4.8 B however was found to have very large experimental errors at day 9 probably due to an aberrant and transient decrease in MCYSTs and NOD.

This study shows that water samples taken from a variety of different sources have naturally occurring microbial populations that are able to degrade cyanotoxins. No comparable degradation occurs in the heat killed controls confirming that loss of toxin is caused by biological degradation with live organisms. These naturally occurring microbial populations present within the water samples tested in this chapter, from Loch Rescobie, Loch Forfar and River Carron, were all found to have some ability to degrade either single or mixed cocktails of cyanobacterial toxins. The least efficient microbial population was, as expected, from the river Carron as this has a high flow rate and no previous history of toxic cyanobacterial blooms.

Although degradation was observed it was slow and the microbial population was only able to degrade single toxins like MCYST-LR and –LF within the 24 day testing period. However compounds like nodularin and complex mixtures of cyanobacterial toxins were not able to be degraded by this microbial population.

In contrast to river water with a high flow rate the lochs sampled have a longer retention time and microbial populations have more time to adapt to compounds persisting within the water. Especially in situations with a previous history of toxic cyanobacterial blooms there was a marked increase in a microbial population's ability to degrade such compounds. This was clearly demonstrated by water from an area with a previous history of toxic cyanobacterial blooms (Loch Rescobie) where toxic compounds like MCYST-LR were rapidly degraded as the findings showed. Other single toxins such as MCYST-LF and Nodularin were also degraded in a similar manor to the findings by (Jones *et al.*, 1994;Lahti *et al.*, 1997;Rapala *et al.*, 1994) where a lag phase was observed before degradation fully commenced. This was more obvious however when the microbial population was subjected to a more complex mixture of cyanobacterial toxins. The type / diversity of compounds a microbial population would be subjected too through out its

history, also plays a major role in their tolerance and ability to adapt to utilize a wide variety of compounds for growth. As in the microbial population form Forfar loch, where no previous history of a toxic cyanobacterial blooms has been documented, but it has a history of non toxic cyanobacteria, it is in the path of a sewage works and has a high migrating bird population. Resulting in a very diverse microbial population, that is quite intriguing as it has a higher affinity for degrading a mixed cyanobacterial toxin, than just individual compounds. It even has a higher ability than that observed in the microbial population from an area with a previous history of cyanobacterial blooms, to degrade such a variety of compounds. Although these MCYSTs and nodularin have been well documented as being chemically stable, it has been published that they are susceptible to biodegradation by naturally occurring bacteria found in rivers and reservoirs (Christoffersen *et al.*, 2002;Jones *et al.*, 1994). These degradative bacteria appear to be present in a wide variety of places as they have been documented in sewage effluent (Lam et al, 1995), lake sediment (Lahti *et al.*, 1997;Rapala *et al.*, 1994;Rapala *et al.*, 1997), lake water (Cousins *et al.*, 1996;Jones *et al.*, 1994;Lahti *et al.*, 1997) and river water(Jones *et al.*, 1994). Also, there is usually an initial lag phase with little or no loss occurring, this

can be as short as a couple of days to even weeks depending on the water body, the concentration of dissolved MCYST/nodularin, climate conditions and even the previous history of the lake (Jones *et al.*, 1994;Lahti *et al.*, 1997;Rapala *et al.*, 1994). However, once the degradation process commences the degradation can be rapid and even complete within a couple of days, but it very much depends on the water body concentration of MCYSTs, microbes and temperature (Jones *et al.*, 1994;Lahti *et al.*, 1997).

Research however, has focused on microbial populations from water bodies with a previous history of toxin producing cyanobacterial blooms, as environments with no previous MCYST exposure may be less likely to posses any specific MCYST degradation pathways within the bacterial population (Holst *et al.*, 2003). Degradation of dissolved MCYST from temperate lakes has been studied in several cases (Rapala et al. 1994), monitored inocula of natural populations of heterotrophic bacteria after exposure to MCYST-LR, from the water phase and sediments from several lakes. Although MCYST-LR has been reported to be degraded over a variety of time periods by heterotrophic bacteria, it is more rapid when the inocula are from locations with a history of cyanobacterial blooms (Rapala *et al.*, 1994). It has also been reported that MCYST-RR was unable

to degrade during a long-term study (months) (Kiviranta et al, 1991 and Watanabe et al. 1992).

Recently (Ishii *et al.*, 2004) furthered the research by (Park *et al.*, 2001)) on an isolated Gram-negative bacterium from the surface water of Lake Suwa and identified is as an aerobic bacillus (rod) and a member of the genus *Sphingomonas*. They discovered that this bacterium 7CY was able to degrade MCYST-LR, -LY, -LW and -LF completely within 4 days from a starting concentration of 6 μg / ml, but was unable to degrade nodularin-Har during the course of the experiments. In contrast, 7CY can degrade nodularin-Har when in the presence of MCYST-RR and both completely degrade within 6 days. This suggests that some compounds may require the presence of others in order for bacterial degradation to occur; it could also be that some compounds induce enzymes during their degradation process that are then capable of utilizing other compounds for nutrition (Ishii *et al.*, 2004). As there are so many potential factors affecting the rate of degradation, major problems occur when comparing various aquatic systems.

When comparing our findings with other research within this area the process becomes very complicated as each microbial population will vary even if it is taken from the same source,

since seasonal variation and climate conditions change yearly effecting the possible microbial proportions or diversity. Therefore comparing the rates of degradation between studies only gives an indication as to whether one microbial population is more efficient or has a higher affinity for toxic compounds than another. If specific bacteria capable of degradation from a microbial population have been isolated, comparing the efficiency of a species will be more reliable and a better indication of how persistent toxins will be when the species in the microbial population has been identified.

The half life's (extrapolated from Figures 4.1 - 4.8) for the degradation of MCYST / NOD both individually or as a mixture in the three waters tested in this study are shown in Table 4.4, with water from Rescobie exposed to only MCYST-LR having a half life of 5 days which is some 2 x faster degradation than that observed in both Forfar and Carron waters. This is comparable to previous research (Christoffersen *et al.*, 2002) where half lives of around 4 days were observed for water with a previous history of toxic cyanobacteria. A similar trend was also observed in the water exposed to only MCYST-LF although the half life was double in comparison to that observed for MCYST-LR it was still around 2 x faster however than the half lifes observed in both Forfar and Carron for the same conditions at 9

days compared to 16 and 21 days, respectively. This is probably due to the fact that the microorganism population of Rescobie has had prior exposure to MCYST-LR and MCYST-LF on numerous occasions due to the *M. aeruginosa* blooms present within this loch. A similar trend was not however observed in the water samples subjected to NOD as both Rescobie and Forfar, have a very similar half life of 15 and 19 days, respectively. This is probably due to the fact that neither population has had prior exposure to this type of hepatotoxin. The population of heterotrophic bacteria present in the river Carron water sample however was found, to be unable to cause any degradation of NOD.

	Compound half-life (days)				
Loch	[D-Asp^3] MCYST-	NOD	MCYST-	MCYST-	MCYST-
Rescobie	RR		LR	LW	LF
MC-LR			5		
MC-LF					9
NOD		15			
4MC+NOD	16	17	13	18	17
Forfar Loch					
MC-LR			10		
MC-LF					16
NOD		20			
4MC+NOD	12	12	13	13	13
River Carron					
MC-LR			11		
MC-LF					21
NOD		N/D			
4MC+NOD	N/D	N/D	>24	>24	N/D

Table 4.4
Degradation of four MCYSTs and nodularin when added in three water samples as either a single compound (MC-LR, MC-LF or NOD) or a mixture (containing 4 MCYSTs and NOD) (N/D is No degradation).

The presence however of a mixture of MCYST / NOD had quite the reverse effect on the trend observed between Rescobie and Forfar as the half lives observed in the Forfar samples were considerably lower than those of Rescobie by ~5 days. Only the MCYST-LR was more comparable as the microbial population has probably had previous exposure to this compound. One possible reason that Loch Forfar gives a shorter half life when mixtures of toxins are present is that it has a diverse microbial population that is more adaptable to stress and the population has been previously exposed to higher levels of contaminants over the years. Also the microbial population of the river Carron water with no previous exposure history to such compounds is totally unable to degrade such compounds when they are in a mixture possibly because of the stress on the bacteria or in an inhibitory effect.

No previous pretreatment or pre exposure studies using MCYSTs and NOD have been documented to have a profound affect on the ability of a microbial population to breakdown such compound. This was why further laboratory experiments were carried out on samples that had previously been exposed in the laboratory to either one or a mixture of MCYSTs / NOD. The half life for the rates of degradation for these experiments is compiled in Table 4.5, where it can be clearly seen that the

microbial population of loch Rescobie is not as capable of degrading a mixture of MCYST/NOD as the microbial population present in the Forfar loch. When comparing the half lives of Loch Rescobie water samples initially exposed to MCYST-LR, -LF or NOD prior to the addition of MCYST / NOD mixture. There was no indication that such exposure increased the microbial population's affinity to these single toxins (MCYST-LR, -LF or NOD) above those with no previous exposure. However prior exposure to the mixture substantially decreases the half life for four out of the five compounds compared to the initial half lives.

Forfar water samples behaved very differently to that of Rescobie, as previous exposure whether to single or multiple toxins, resulted in them all behaving in similar manor. The average half life for all five compounds in the four different pretreatments is ~5 days at least half than that observed for initial exposure rates.

Pre-exposure[a]	Compound half-life (days)				
Loch Rescobie	[D-Asp^3] MCYST-RR	NOD	MCYST-LR	MCYST-LW	MCYST-LF
MC-LR	8	>15	13	>15	>15
MC-LF	11	14	>15	>15	>15
NOD	7	12	>15	>15	14
4MC+NOD	8	11	10	12	10
Forfar Loch					
MC-LR	5	5	11	6	6
MC-LF	5	5	5	6	5
NOD	5	4	5	5	5
4MC+NOD	5	5	5	5	5
River Carron					
MC-LR	>15	>15	>15	>15	>15
MC-LF	>15	>15	>15	>15	>15
NOD	>15	>15	>15	>15	>15
4MC+NOD	>15	>15	>15	>15	>15

Table 4.5
Degradation of four MCYSTs and nodularin when added in three water samples that have been pre-exposed to either a single compound (MCYST-LR, -LF or NOD) or a cocktail containing four MCYSTs and nodularin (N/D is No degradation).[a] Each sample was pre-exposed to the compounds or cocktail for a period of 30 days prior to the addition of a mixture of four MCYSTs and nodularin.

These findings clearly identify that the microbial diversity of a water body not only can improve its toxin degradation ability but also that prior exposure to multiple toxins increases a population's ability to degrade complex compounds. Compounds like MCYST/nodularin are well documented to have toxic side affects on mammals however they have also been shown to cause an inhibitory effect on microbial respiration as indicated by reduction in bioluminescence of some bacteria (Lawton *et al.*, 1990). This inhibitory effect may also occur in many other bacteria even those able to carry out biodegradation if the levels

are high enough or if more than one variant is present.

Further work in this area should focus on the chemistry of micro organisms and / or consortia of microbes that are capable of degrading MCYST with the view to their exploitation in water treatment.

Chapter 5

Photocatalysis of MCYST

5.1 Introduction

Increasing concern about cyanotoxins in drinking water has prompted research to evaluate the effectiveness of current water treatment approaches and to develop new method of treatment. Previously, research has identified that photocatalysis using TiO_2 and ultraviolet (UV) light effectively removes MCYST-LR and other MCYSTs effectively from water (Robertson *et al.*, 1997) even at concentrations of 200 µg / ml. MCYSTs were reported to be undetectable by HPLC within a 40 min period. This approach to water treatment also effectively degrades many other unwanted compounds that may occur in drinking water (Eggins *et al.*, 1997;Mills *et al.*, 1996). Research found that TiO_2 in water under illumination of UV light was able to degrade a wide range of organic pollutants e.g. humic substances. The TiO_2 produces oxidizing radicals which are believed to degrade compounds as they are more oxidizing than any other reagents currently being used in potable water treatment (Robertson, 1996).

TiO_2 has been well documented in many photocatalytic studies, with a relatively high reactivity and chemical stability under UV light that is greater than the bandgap energy of the catalyst

(wavelength λ <380 nm). This causes electrons and holes to be produced in the conduction and valence bands respectively, with the electrons having a highly reactive reduction potential while the holes have a highly oxidative potential and together cause catalytic reactions on the catalyst surfaces namely photocatalytic reactions (Anpo, 2000). Photocatalysis has also been referred to as artificial photosynthesis but unlike photosynthesis it is only able to make use of 3 - 4 % of solar beams that reach earth (Anpo, 2000). Therefore the development of photocatalysts that are able to yield high reactivity under visible light (λ >380 nm) is highly desirable in order to utilize the main part of the solar spectrum. This would result in photocatalysts having a much higher application potential, potentially giving an environmentally sustainable way of chemically cleaning pollutants or pollution. The ability to utilize solar energy much more efficiently also would result in lower running costs in comparison to UV catalysts that require light of a specific wavelength to bridge the bandgap. Restricting use to UV activation makes their use in large scale treatment processes (water) very costly, especially when there is an abundance of natural energy available (solar energy) that could be used if visible light catalysts were as effective.

While TiO_2 / UV has proved extremely successful at removing

MCYST there is some resistance to full exploitation possibly due to the cost of providing UV light which is energy demanding. Research into visible light activated catalysts is therefore very important to future exploitation of TiO_2 in water treatment. Although the effectiveness of TiO_2 (P25 Degussa) and UV illumination has been well documented as an effective method of degrading many unwanted compounds dissolved in potable water the cost of implementing and running such a system on a large scale can be prohibitive and research into new and novel catalysts is ongoing.

This chapter presents studies carried out using novel visible light catalysts, to determine if they have the potential to degrade MCYST-LR under visible light illumination. Their ability and effectiveness was compared to previous documented research on UV photocatalysts. Ideally their rates of photocatalysis have to be comparable with those of UV catalysts but with the lower energy demand of using visible light or even sunlight.

5.2 Methods

5.2.1 Visible light activated catalysts

Novel visible light activated catalysts were developed by Prof. Horst Kisch (Institute Für Anorganische Chemie, Universität Erlangen-Nürmberg, Erlangen) which incorporated specialised dopants the nature of which is commercially sensitive. In total four newly developed catalysts (KSHGL-104/9765, KSH-AYYQ-PT, TH New and KSH Burg) were irradiated with visible light and their photocatalytic activity analysed. Degussa P25 (a known UV photo catalyst) was used as a control throughout all the visible light experiments as photocatalysis only occurs when exposed to wavelengths (λ) in the UV spectrum (Robertson *et al.*, 1997). Any photocatalysis observed under control conditions would indicate the presence of wavelengths ≤380 nm and a possible failing in the experimental design.

5.2.2 Preparation of MCYST-LR solution

The cyanobacterial toxin MCYST-LR was extracted from cells of *M.aeruginosa* PCC7820 (Pasteur Institute, Paris, France) by the Cyanobacterial research Group (The Robert Gordon University, Aberdeen, UK). Four vials containing 1.6 mg of dry frozen (-20

ºC) MCYST-LR (99 % pure) was reconstituted in Milli-Q water through a process of sequential washing (where 1 ml was added followed by 1 min vortexing) ten times. Each vial was then washed a further two times with 10 ml of Milli-Q water and vortexing until a final volume of 120 ml with a concentration of 0.05 µg / ml was achieved. The solution was then stored at 4°C in an air tight 150 ml glass bottle and used to carry out experiments; with 5ml aliquots removed and allowed to reach room temperature prior to experiments being carried out.

5.2.3 Photocatalysis of MCYST-LR by visible light catalysts

The sample was placed in a 20 ml glass vial with constant stirring at a distance of 30 cm from the front of the light source (standard 500 W Halogen outdoor light) protected by a UV filter (200mm square, Cambridge instruments) giving a light intensity of 393 µmol s^{-1} m^{-2} (Figure 5.1). The lamp was switched on for 30 min prior to experiments; a sample was removed (150 µl) from the starting solution of MCYST-LR prior to the addition of catalyst (T_0 sample). The catalyst (1 % w/v),(Robertson *et al.*, 1997) and MCYST solution were mixed for 5 min to equilibrate and a sample removed prior to exposure to the light source (T_{0+Cat} sample). The reaction mixture was illuminated and a

sample (150 µl) removed at 10 min intervals for the first 60 min and at 20 min intervals there after until 120 min was reached. On removing the samples they were immediately centrifuged (12,000 x g) for 5 min and then 100 µl of the supernatant removed for analysis by HPLC. Samples were stored in the dark prior to analysis by HPLC (Section 2.2.6).

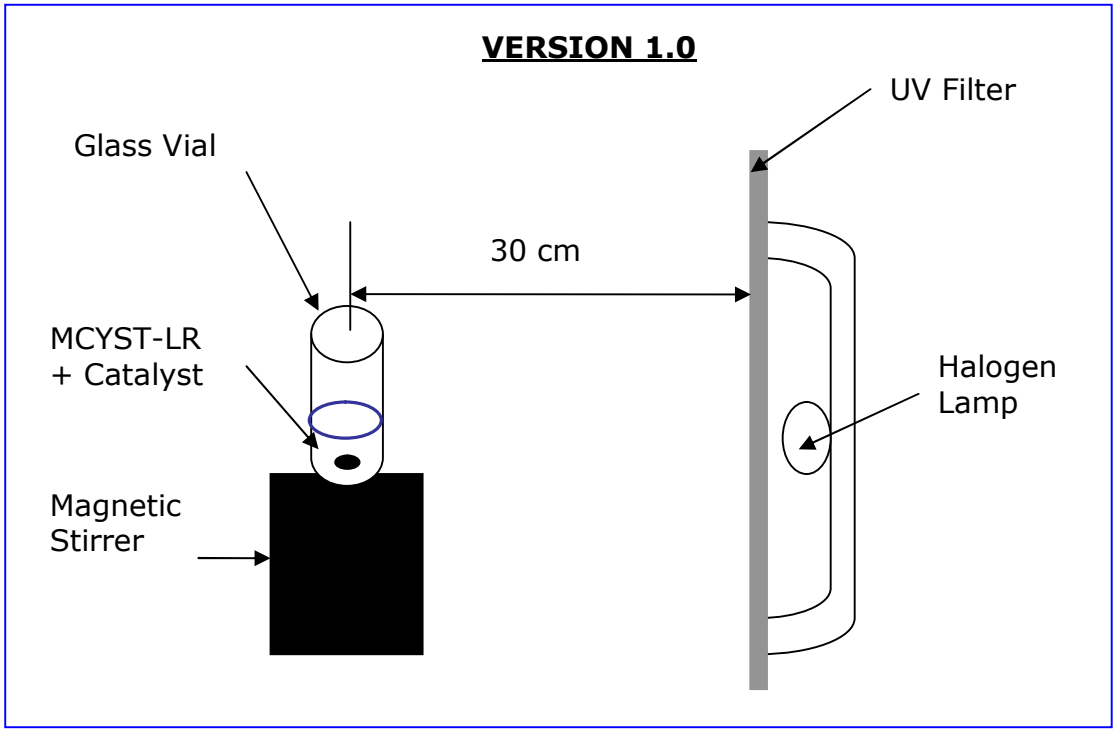

Figure 5.1
TiO_2 Photocatalysis Version 1.0 in which a halogen light source (393 µmol s^{-1} m^{-2}) with UV filter provided constant illumination to a solution of MCYST-LR (0.05 µg / ml) which contained catalyst (1 % w/v) under constant stirring.

5.2.4 Photocatalysis of MCYST-LR by visible light using a UV and IR filter

Changes were made to Version 1.0 of the photocatalytic reaction apparatus by the addition of an IR filter (from a 1980, Perkin Elmer instrument) in front of the UV filter to limit temperature variation between the start and end of the experiment. Also a fan was introduced to dissipate the heat from the sample during the 2 hr experiment (Figure 5.2, Version 2.0); all the other parameters remained as detailed in section 5.2.3 using MCYST-LR solution prepared as per section 5.2.2.

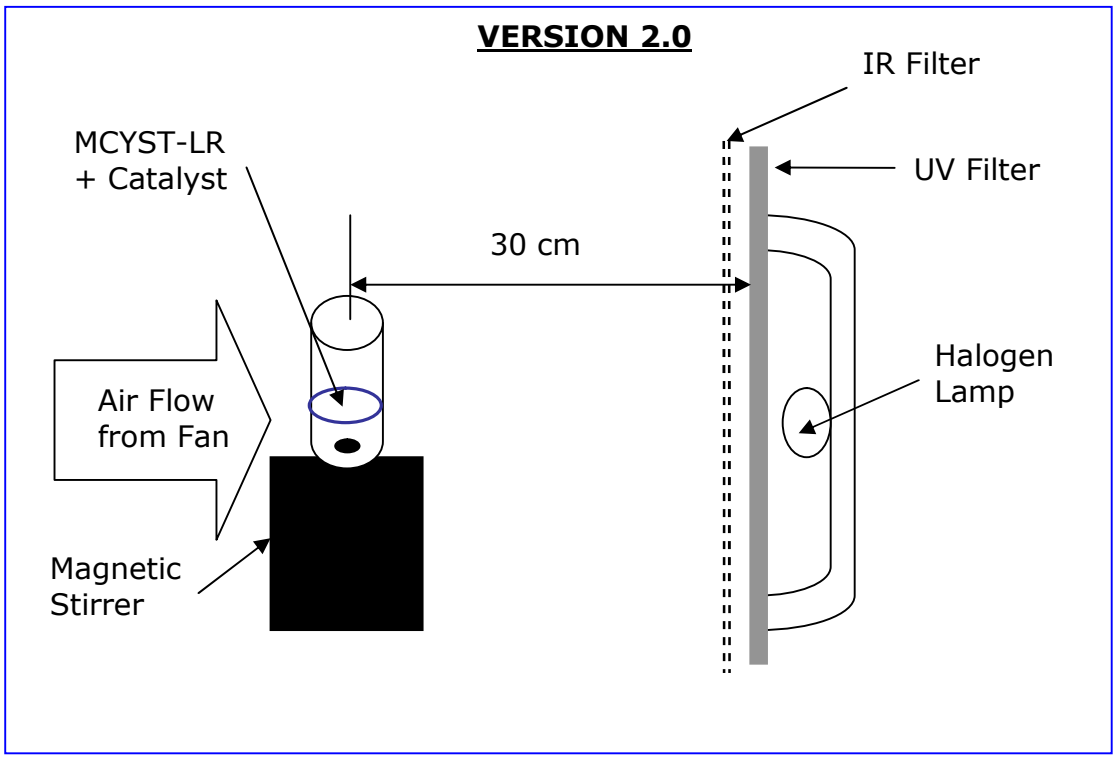

Figure 5.2
TiO_2 Photocatalysis Version 2.0 using a halogen light source (393 µmol s^{-1} m^{-2}) with IR and UV filters and a solution of MCYST-LR (0.05 µg / ml) containing catalyst (1 % w/v) under constant stirring and cooled by a desk fan.

5.2.5 Catalytic degradation using a liquid UV filter

Photocatalytic setup Version 2.0 was further modified by the addition of a sodium nitrite (2%) liquid filter surrounding the sample. This absorbed any remaining UV light not already removed by the solid UV filter directly in front of the light source. It also provided an additional temperature control; reducing the possibility of heat induced catalytic activity, in addition to the air cooling already in place during the 2 hr experiment (Figure 5.3). All the other parameters remained as detailed in section 5.2.3 and using MCYST-LR 0.05 µg / ml.

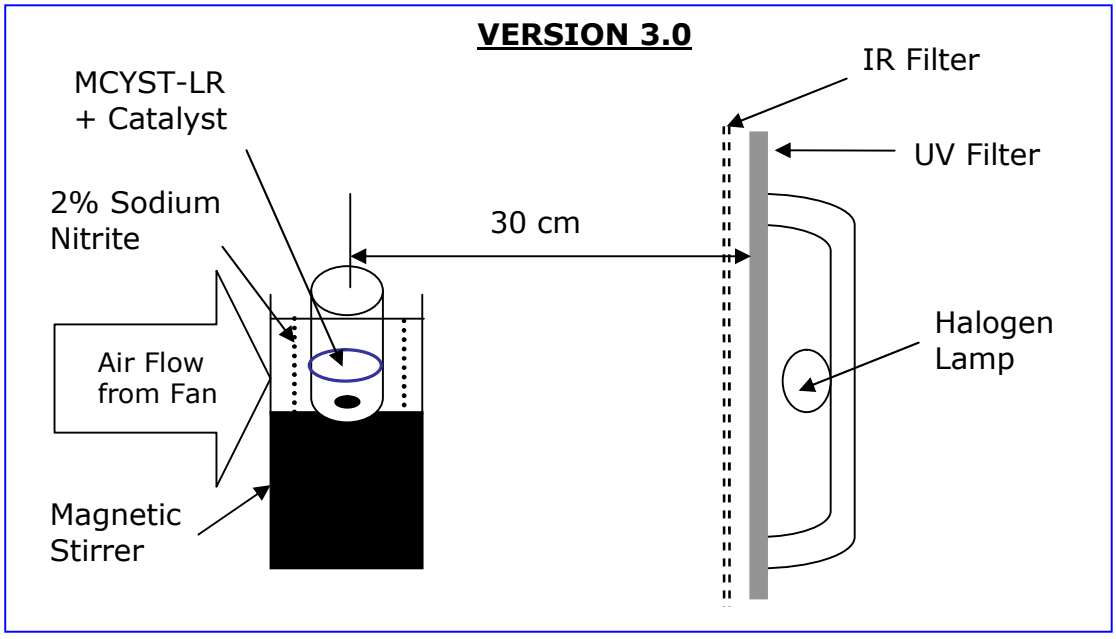

Figure 5.3
TiO_2 Photocatalysis Version 3.0 used a halogen light source (393 µmol s^{-1} m^{-2}) with IR and UV filters and a solution of MCYST-LR (0.05 µg / ml) containing catalyst (1 % w/v) under constant stirring. With the sample surrounded by a 2% solution of sodium nitrite to absorb remaining UV not removed by the solid filters and cooled by a desk fan.

5.2.6 Photocatalysis under various light intensities

The ability of visible light catalysts to degrade MCYST-LR was then tested further by using version 3.0 setup and altering the light intensity. This was achieved by altering the distance between the light source and the sample to 30, 60 and 90 cm resulting in the light intensity changing from 393, 117 and 53 μmol s^{-1} m^{-2} respectively as the distance increased. The light intensity decreased with distance following the reported behaviour where light intensity is proportional to the distance squared (Newman, 2007).

Intensity $I = 1/R^2$

5.2.7 Evaluation of photocatalysis of MCYST-LR by modified P25 under UV light

Degussa P25 and modified Degussa P25 (modified by laser treatment so additional Ti(III) defects are introduced into the matrix as well as a greater percentage of rutile material (approximately 50% rutile / 50% anatase as opposed to the 25% rutile and 75% anatase in P25 (Robertson *et al.*, 1997)) catalyst were tested for their ability to degrade MCYST-LR in the presence of UV light using the following set up shown in figure 5.4. This was to determine if laser modifications to P25 would alter degradation rates significantly compared to those observed with standard P25 under UV light.

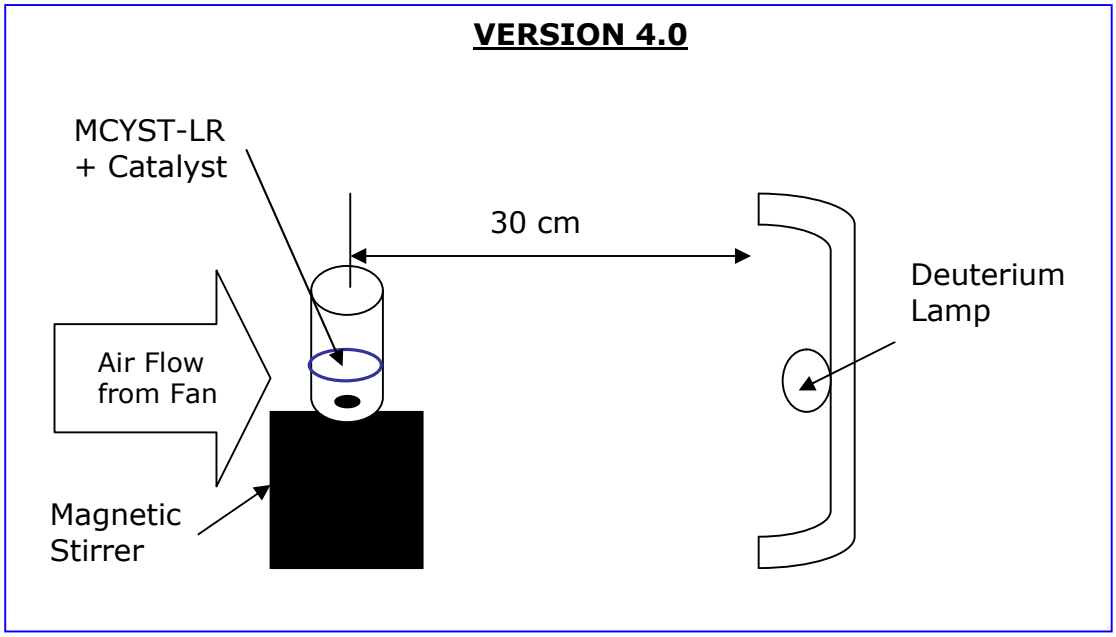

Figure 5.4
TiO_2 Photocatalysis Version 4.0 using a deuterium light source with a solution of MCYST-LR (0.05 µg / ml) containing catalyst (1 % w/v) under constant stirring.

5.3 Results and discussion

5.3.1 Degradation of MCYST-LR

Visible light catalysts that have been tested in this chapter may be the next step in environmentally sustainable chemical cleaning; therefore it was imperative that the experimental design only allowed exposure to visible light and not UV. It was also deemed necessary to test each experimental design using a control sample of TiO_2 (Degussa P25) as visible light is unable bridge the bandgap required for photocatalysis to occur. Initial experiments used version 1.0 in which two new catalysts were tested along with the control (Deggusa P25) (Figure 5.5). The effect of dark adsorption (adsorption of compound by the catalyst) was clearly observable which meant a proportion (about 20%) of MCYST-LR adsorbed to the surface of the catalyst changing the detectable level from T_0 to T_{0+cat} , with further decreases from T_{0+cat} as a result of degradation and not dark adsorption. The most significant degradation was observed by catalyst KSH AYYQ-PT as only 28 % of MCYST-LR remained after 20 min. As for the other two catalysts P25 and KSH 104/9765 after 20 min 54 and 70 % remained respectively. The observation that degradation occurred for P25 suggests that not all the UV light had been eliminated. This experimental design

was not capable of stopping all the UV light from reaching the test condition as bridging of the bandgap must have been occurring in order for degradation of MCYST-LR to occur in the P25 condition.

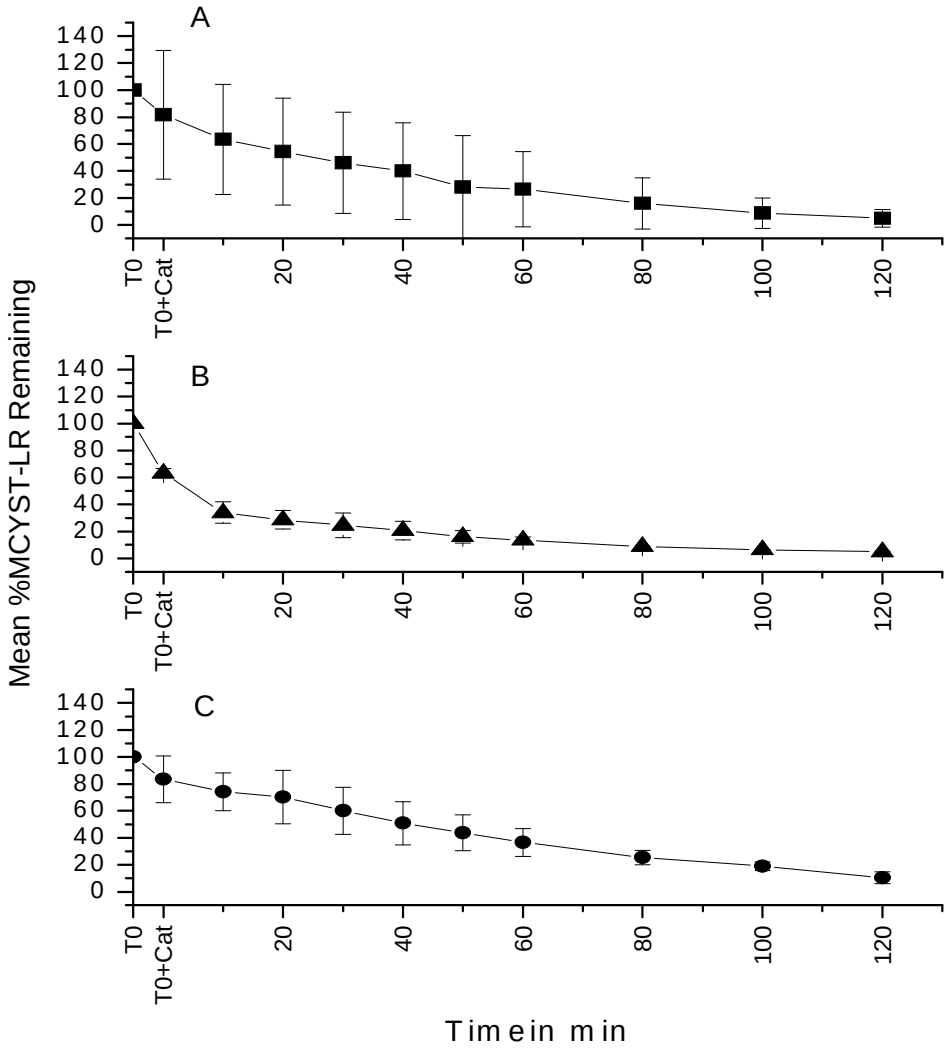

Figure 5.5
Photocatalytic degradation of MCYST-LR in the presence of P25 (A), KSH AYYQ-PT (B) and KSH 104/9765 (C) using halogen light at an illumination of 393 μmol s^{-1} m^{-2} filtered through a UV filter (Version 1.0).

Therefore further tests were carried out using P25 and the system was modified by addition of an IR filter Version 2.0, to eliminate sample heating which also might have affected the stability and rate of MCYST degradation. Therefore P25 catalyst was tested using the same light intensity (393 μmol s^{-1} m^{-2}) only with the presence of UV and IR filters between the sample and the light source. However the rate of degradation with P25 catalyst over a 20 min period actually increased to leave only 34 % MCYST-LR remaining after 20 min (Figure 5.6, A), for this reason the P25 catalyst was then tested under dark conditions to determine if the catalyst was reacting to the solution of MCYST-LR with no activating light source (Figure 5.6, B). Under dark conditions there was no catalytic activity observed as expected, suggesting that UV light was passing through to the sample even with both filters was present. The only other source would be from the surrounding environment in which the experiment was being carried out; therefore the experiment was repeated using P25 and MCYST-LR solution under constant stirring on the open bench with no light on. The result from this experiment in (Figure 5.6, C) clearly shows that under normal laboratory bench conditions the P25 catalyst is unable to breakdown MCYST-LR. This result proved that the experimental set up in Version 2.0 was unable to eliminate all UV light

reaching the sample being tested at 30 cm from the light source.

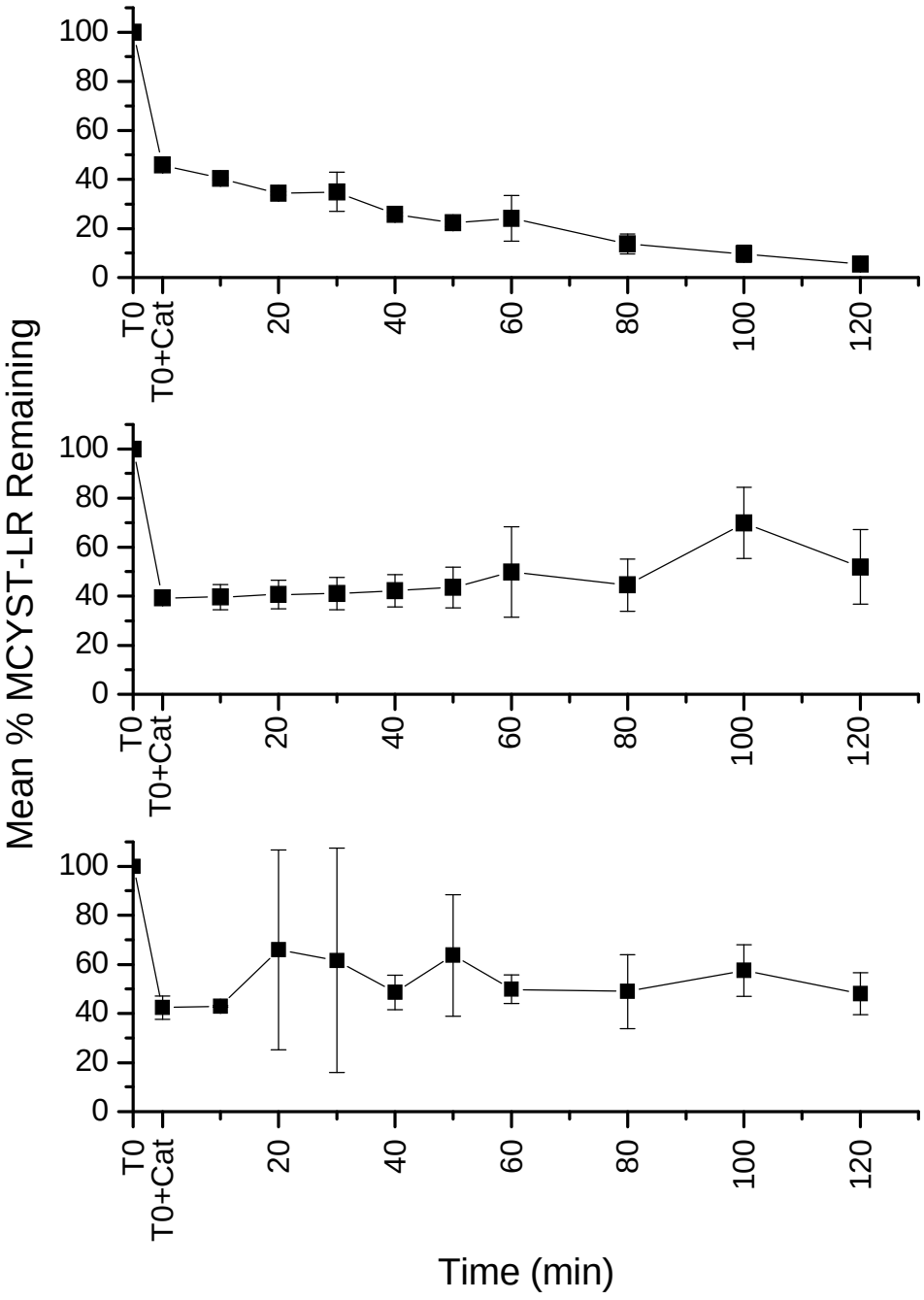

Figure 5.6
Photocatalytic degradation of MCYST-LR in the presence of P25 ■ (A) halogen light at an illumination of 393 μmol s^{-1} m^{-2}, (B) Dark conditions with no illumination and (C) Standard laboratory bench conditions with no halogen illumination (Version 2.0).

Further UV filtering was achieved through the introduction of a liquid filter (2 % sodium nitrite) as in Version 3.0 to absorb any residual UV, preventing it from activating the catalysts. After introducing the liquid UV filter the system was tested with P25 catalyst in the presence of 393 µmol s-1 m^{-2} illumination from the halogen light, after initial dark adsorption 46% of MCYST-LR remained detectable. The sample was subjected to a period of 120 min exposure with samples taken at the time points stated in (Section 5.2.3). The results were plotted in (Figure 5.7) and fluctuations between time points were considered to be as a result of equilibration between MCYST-LR and the catalyst not degradation as previously observed in other experiment.

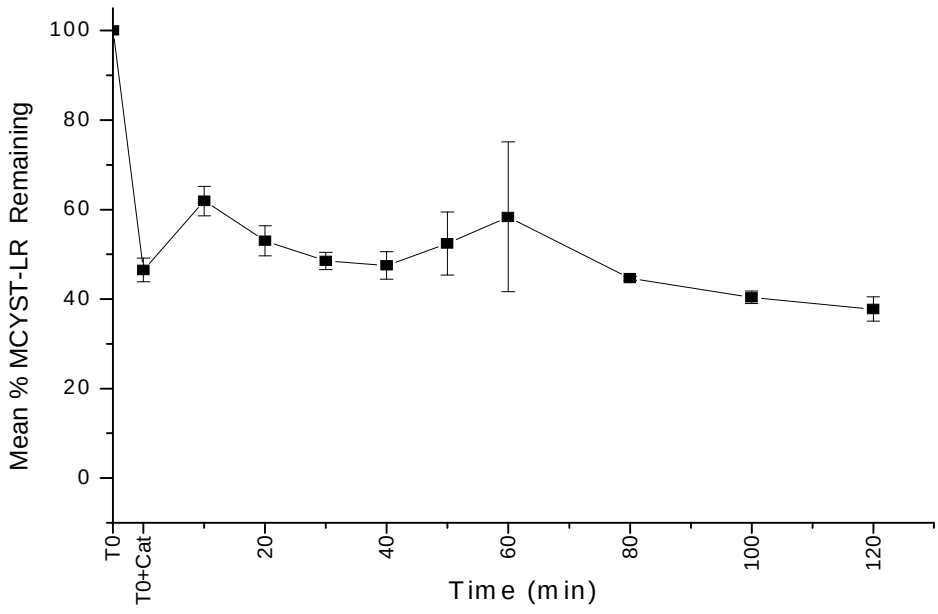

Figure 5.7
Photocatalytic degradation of MCYST-LR in the presence of P25 ■ using a 2 % sodium nitrite UV filter and 393 µmol s^{-1} m^{-2} illumination by the halogen light source with a solid UV and IR filter in place (Version 3.0).

This experiment proved that no UV light activation could occur in the system and therefore testing for visible light activation of the novel catalysts could proceed with confidence that any observed degradation was in fact due to visible light activation. Four catalysts were then tested using the set up detailed in Version 3.0 and the results are shown in Figure 5.8, with P25 catalyst was used as a control as no degradation of MCYST-LR should occur if there is no UV light present. This was the case, as no degradation of MCYST-LR was observed in the P25 catalyst test (P25 control results not shown). The results in Figure 5.8 clearly show how different catalysts have different adsorptions, resulting in variation of T_{0+Cat}. KSH-AYYQ-PT (PT) has the highest initial binding ability with 83 % followed by KSH-Burg 58%, P25 54%, KSH-GL-104/9765 37% and finally THNew 20%. The rates of degradation from T_{0+Cat} also varied quite considerably with the fastest rate of degradation caused by KSH-Burg where less than 10% remained after 20 min of exposure to visible light. It also achieved complete degradation within 60 min, compared to KSH-AYYQ-PT and KSH-GL-104/9765 where 8 and 19% remained after 60 min respectively. The THNew catalyst was considerably slower and after 60 min 71% of MCYST-LR remained and after 120 min more than half of the MCYST-LR still remained.

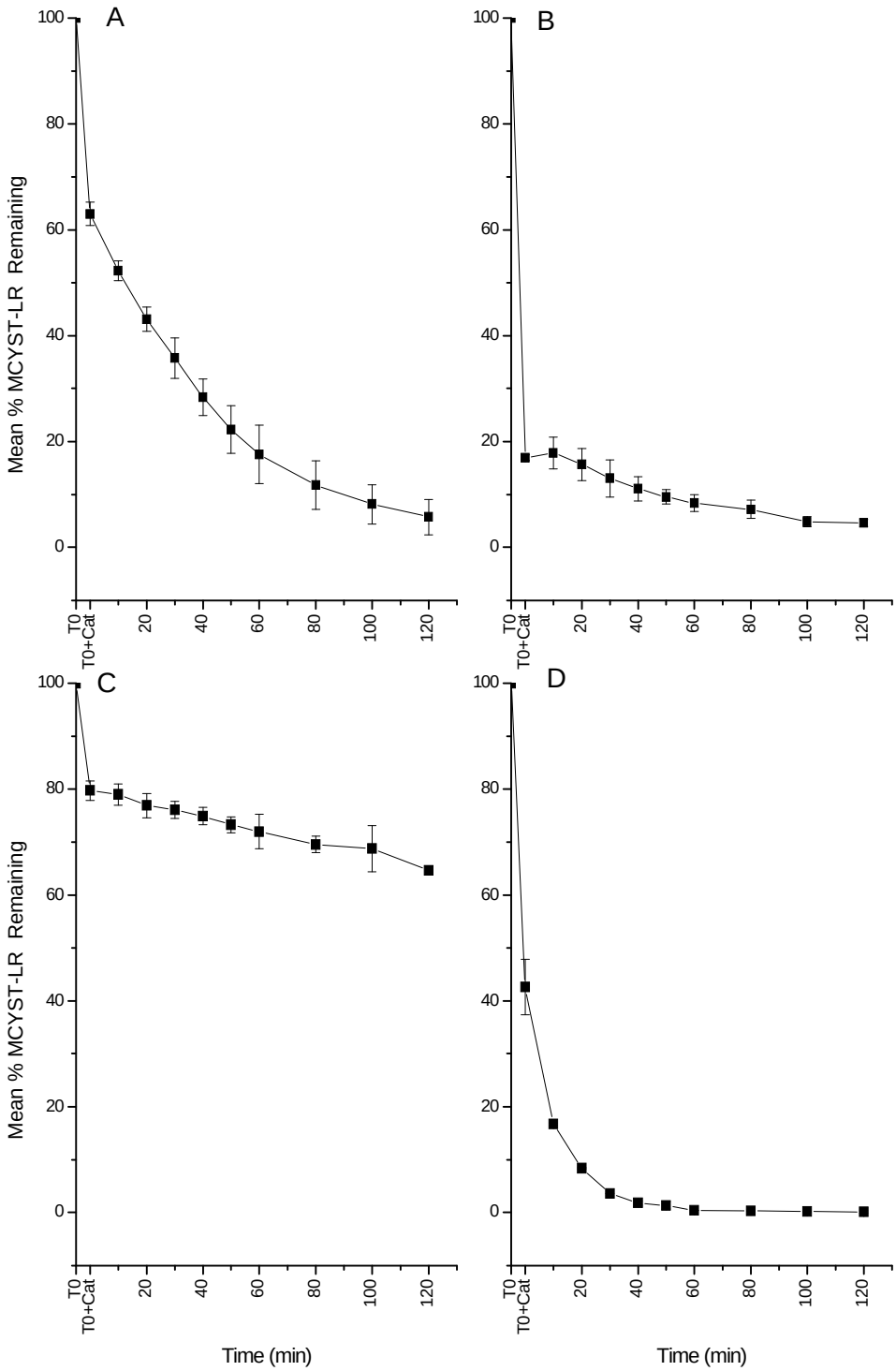

Figure 5.8
Photocatalytic degradation of MCYST-LR by four new visible light catalysts; KSH GL 104/9765 (A), KSH-AYYQ-PT (B), THNew (C) and KSH Burg (D), showing the percent remaining after 120 min of exposure to visible light at 393 μmol s^{-1} m^{-2} (Version 3.0).

The catalysts KSH GL 104/9765 and KSH Burg were however very impressive degraders and significant photocatalysis occurred during the 120 min of the experiment using the version 3.0 system. However their rate of degradation varied quite considerably with KSH GL 104/9765 being much slower although it was consistently dropping over time it was unable to match the rate of KSH Burg which reached 100% photocatalysis within 40 min. Such a rate of photocatalysis by the visible light KSH Burg is almost comparable to the rate of degradation observed under UV illumination for P25.

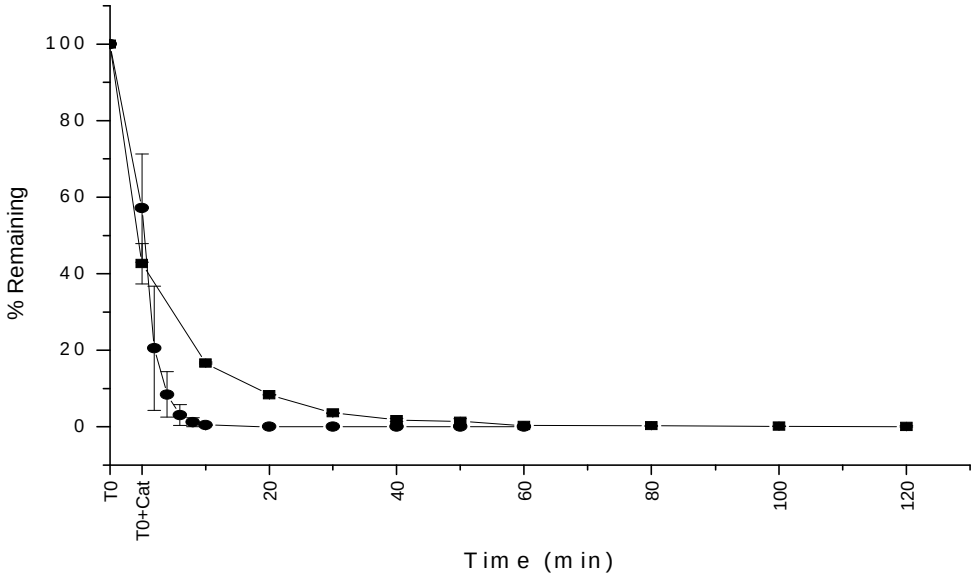

Figure 5.9
Comparison of the photocatalytic degradation of MCYST-LR in the presence of KSH Burg ■ under visible light illumination by a halogen light source (version 3.0) and P25 ● under UV illumination by a deuterium light source (Version 4.0).

The main difference between these being that after 10 min in the presence of P25 under UV light 100 % of MCYST-LR had been photocatalysed and <20 % of MCYST-LR still remained in the KSH Burg (visible light) samples from a starting concentration of 50 µg / ml (Figure 5.9).

As the most efficient catalytic activity was observed by KSH-Burg, further work was carried out at a lower light intensity of 53 and 117 µmol s^{-1} m^{-2} in order to determine how this would affect the rate of degradation. This experiment was set up as per Section 5.2.6 and the results are shown in Figure 5.10, where the results show that as the intensity of visible light decreases the rate of degradation decreases but not substantially. The time required to reach just 20 % MCYST-LR remaining, also reflects the change to the rate of degradation as it took 11, 19 and 33 min for 393, 117 and 53 µmol s^{-1} m^{-2} light intensity, respectively. Therefore the rate of catalytic degradation by KSH-Burg is directly related to the intensity of visible light available.

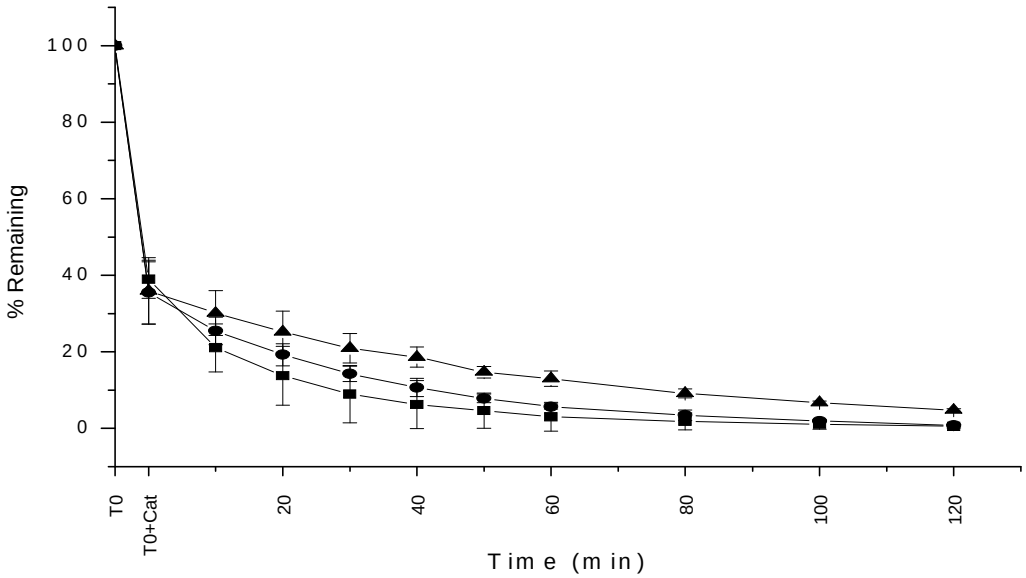

Figure 5.10
Photocatalytic degradation of MCYST-LR in the presence of KSH-Burg at 393 µmol s^{-1} m^{-2} ■, 117 µmol s^{-1} m^{-2} ● and 53 µmol s^{-1} m^{-2} ▲ illumination by a halogen light source with a liquid/solid UV and IR filter in place (Version 3.0).

5.3.2 Degradation of MCYST-LR using modified P25 and UV light

The last catalyst to be tested was a modified P25 catalyst, although P25 has already been well documented for its ability to degrade MCYST-LR in the presence of UV light. A new modified P25 (Section 5.2.7) was tested to compare the rates of photo degradation to that of P25 and determine if the modifications have improved or deteriorated the TiO_2 degradation potential. Both catalysts were tested using the experimental design as per

Version 4.0 with a deuterium lamp the rate of degradation for both the modified P25 and P25 showed no significant differences (Figure 5.11).

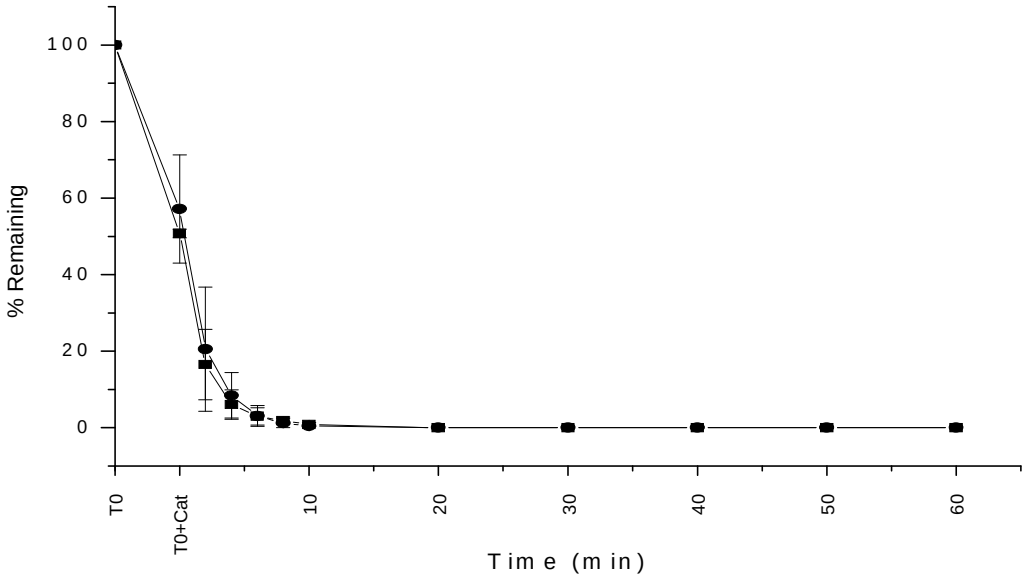

Figure 5.11
Photocatalytic degradation of MCYST-LR in the presence of modified P25 ■ and P25 ● under UV illumination by a deuterium light source (Version 4.0).

In this study the successful investigation using version 3.0, where samples were only subjected to visible light and not UV. Any photocatalytic degradation caused by the new catalysts was as a direct result of the visible light being greater than the bandgap energy for these new catalysts and not from UV irradiation. The four new visible light catalysts that were tested in this chapter all showed some photocatalytic ability, but the

rates of photodegradation by KSH-AYYQ-PT and TH New were less than favourable and although the dark absorption in each is very different from very low to very high neither have a bandgap that is able to utilize wavelength $\lambda > 380$ nm.

Although photocatalysis research started in the early 1970s and a wide variety of applications such as self cleaning glass, anti-bacterial, anti-viral, fungicidal, anti-soiling, self cleaning deodorizing, air-purification, water-treatment and water-purification have been suggested; it has really been the environmental applications that have stimulated the development of photocatalysts. The main photocatalyst has been TiO_2 because of its stability under UV light and lack of toxicity (even in large quantities). However, TiO_2 has been hampered by its wide bandgap (3.2 eV) from widespread applications due to its requirement for UV radiation ($\lambda \leq 380$ nm) required for photocatalytic activation as only a small proportion of the solar spectrum consists of UV light. Using UV light has many problems associated with it; along with high running costs there are the practical difficulties of providing large or sufficient quantities for industrial applications and it cannot be used without potential hazards to the operator.

This has increased attempts to shift the onset of the TiO_2 destruction from UV to the visible region by doping (addition of

functional groups, transition metals or Hydrogen) the catalyst with transition metals or by hydrogen reduction (Kumar *et al.*, 2005). In addition to the transition metals other attempts have been made to photosensitize TiO_2 with a variety of coloured organic (dyes) and inorganic chemicals, albeit with very limited success (Kumar *et al.*, 2005). The implantation of metal ions identified that it was possible to enable a lowering of the energy for the conductance band for TiO_2. The comparative sequence of metal ions V>Cr>Mn>Fe>Ni correspond with their effectiveness in shifting the photocatalytic response to the visible light irradiation (λ > 450 nm)(Kumar *et al.*, 2005). However metal ions of Ar, Mg or Ti had no effect of the conduction band energy requirements and the development of a catalyst capable of lowering the conductance band to give effective photocatalysis may prove difficult. Although more recently ground breaking studies have produced $TiO_{2-x}N_x$ films that react to visible light and have similar photocatalytic activity to that of TiO_2 under UV light, resulting in renewed interests in visible light photocatalysis (Kumar *et al.*, 2005).

Although extensive research has be carried out using UV photocatalysts the development of effective visible light photocatalysts is still very much in its infancy and very little research has been carried out on their photocatalytic ability of

environmental contaminants as studied in this chapter. KSH Burg and KSH GL 104/9765 are shown to have the ability to photocatalyse MCYST-LR by visible light activation of the new catalyst bandgap within the 120 min analysis period. This is the first report of MCYST TiO_2 photocatalysis using visible light activated catalysts. It was also noted that variations in the visible light intensity did not substantially affect the rate of photocatalysis. Even the lowest light intensity of 53 μmol s^{-1} m^{-2} reached over 95% degradation within the 120 min analysis time period suggesting moderate light intensity is sufficient for effective degradation. This type of result with varying light intensity on the photocatalytic ability of visible light activated catalysts has not been documented to date and is a major concern when dealing with solar energy as light intensity will fluctuate and an activation range would really need to be investigated further.

In conclusion the future of visible photocatalysis is very much in its infancy and there are still major developments that need to be investigated, although the results from the new catalysts tested in this chapter are very promising. Further research into these visible light catalysts and their mode of action would be very beneficial and ideally determine if there is a maximum concentration and how they deal with a mixture of complex

environmental pollutants. Research by (Chen *et al.*, 2007;Moon *et al.*, 2003;Sun *et al.*, 2005) all indicated that deposition of platinum on TiO_2 promoted the optical absorption into the visible region making it suitable for excitation by visible light. Other areas for further investigation are how temperature and pH affects visible light photocatalysis. The potential and effectiveness of these catalysts has wide reaching implications, as future regulations on pollutants in the environment become more stringent it is essential to develop more efficient and less harmful methods of treatment. Further research in this field would be beneficial in understanding the true potential for large scale development, possibly in areas such as water treatment. As this study has also only focused on the degradation of one MCYST studies on the effective degradation of other such compounds would also prove very beneficial.

Chapter 6

Conclusion

6.1 Concluding remarks

This research initially set out to investigate and develop an understanding of the toxic secondary metabolites (MCYSTs) produced by cyanobacteria. In particular the focus was on cultures of *M. aeruginosa* PCC7820 known to produce four MCYST variants whose function has yet to be elucidated. Many theories exist as to why toxic strains are usually more prolific, ranging from protection against grazers or potentially compounds used to facilitate the uptake of nutrients from their surrounding environment under limited conditions.

This study identified that increasing inorganic carbon through the addition of $NaHCO_3$ caused significant reductions in the level of MCYST, with further investigations eliminating the possibility that the effects were not as a result of the elevation in Na^+ ions. Although to truly measure the effects of increased inorganic carbon minimal interference from either buffers or other parameters is preferential. Direct sparging with air enriched with 5% CO_2 was the preferred method to provide increased inorganic carbon. The experimental design was crucial utilizing standard medical materials (Cannula) to assist in improving sampling and reducing potential for contamination. Automation

also provided the ability to accurately carry out pulse experiments over various time periods and reduce analytical variation. Accuracy, quality and consistency all provide confidence in the results. This study however found that direct sparging with air enriched with inorganic carbon increased MCYST levels even when expressed as a proportion of the net protein, indicating that selective synthesis occurs in favour of MCYSTs. Analysis of a range of parameters (e.g. cell numbers, protein and biomass) proved beneficial in understanding certain activities either directly or indirectly. This study identified increasing the level of inorganic carbon with air enriched with 5% CO_2 resulted in an increase in MCYST-LR, -LW and –LF levels produced in *M. aeruginosa* PCC7820. While levels of MCYST-LY were decreased, further research is now required to evaluate the regulatory mechanisms affected by increased CO_2 availability as cultures seam to become more toxic in elevated CO_2 conditions. Recent progress in the use of transcription of the MCYST genes may be relevant to this work. Especially with respect to global climate change and the threat of fresh waters becoming increasingly eutrophicated it is essential that further studies are undertaken to evaluate the combined effect of elevated nutrients, temperature and CO_2. Interestingly in this research altering the form of available inorganic carbon from bicarbonate

to CO_2 altered the levels of MCYSTs present in cultures of *M.aeruginosa* PCC7820. As the presence of $NaHCO_3$ decreases MCYST levels and CO_2 increases MCYST levels but from our findings increasing the Na^+ ions had no affect on MCYST levels.

The persistence of these MCYST secondary metabolites in the environment is a potential problem as persistent compounds usually require elaborate and complicated methods of treatment that are inevitably costly. The process of natural degradation has only recently had attention as more research groups try to identify species capable of such breakdown with the potential to develop them possibly commercially or understand if they can be used safely to treat acutely contaminated waters. It also helps in the development of models to predict how long natural degradation may take and calculate the advantages of treatment over that of natural degradation. This research identified that the prior history of a water body and exposure to stable complex compounds can alter a microbial populations degradation potential. In particular water taken from and area with no prior history of cyanobacterial blooms but down stream of sewage treatment, had higher degradation potential than those only previously exposed to cyanobacterial blooms. Therefore pretreatment or prior exposure plays a major role in shaping the

microbial populations within water bodies, although altering such natural microbial diversity in order to achieve new water treatment regulatory levels may not always be the best option. The environmental impact or affect on other life forms needs extensive investigation as damage can be irreparable if non native species are introduced, developing an efficient but controllable method would seam a more practical solution.

Although natural degradation is certainly an ideal way for the disposal of such toxic compounds the development of an advance treatment approach may be necessary. TiO_2 photocatalysis has relatively recently been explored for industrial application of water management but require costly UV light which may have reduced the take up of this technology. Therefore recent work developing new and novel catalysts which have equal or better activity at degrading such compounds as MCYSTs are of great interest. While using only visible light as the activation energy and not UV, this study has identified a few novel visible light catalysts which rival the degradation potential of Degaussa P25 under UV light. Further work will now be carried out to determine the performance of these novel catalysts in natural waters.

It may be predicted that the health hazards associated with toxic cyanobacterial blooms will be of increasing concern in years to come especially with pressure on the availability of freshwater. A good understanding of the factors influencing bloom toxicity and ways to remediate the problems (e.g. biodegradation and / or water treatment) will be essential to support water resource management.

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